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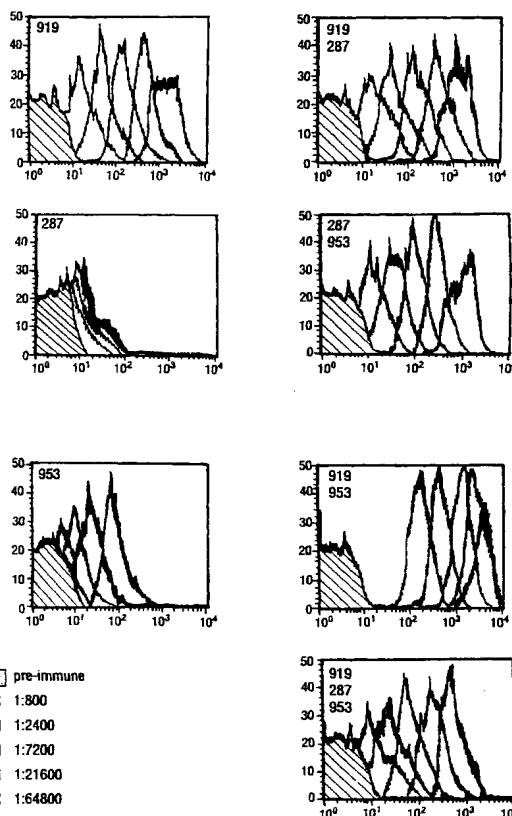
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(54) Title: COMBINATION NEISSERIAL COMPOSITIONS



(57) Abstract: Compositions comprising a first biological molecule from a *Neisseria* bacterium and a second biological molecule from a *Neisseria* bacterium. The term "biological molecule" includes proteins and nucleic acids. Preferred *Neisseria* species are *N. meningitidis* and *N. gonorrhoeae*.

WO 00/71725 A2



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## COMBINATION NEISSERIAL COMPOSITIONS

All documents cited herein are incorporated by reference in their entirety.

### FIELD OF THE INVENTION

This invention relates to compositions comprising combinations of biological molecules from  
5 *Neisseria* bacteria, particularly *N.meningitidis* and *N.gonorrhoeae*.

### BACKGROUND ART

*Neisseria meningitidis* and *Neisseria gonorrhoeae* are non-motile, Gram negative diplococci that are pathogenic in humans.

Based on the organism's capsular polysaccharide, 12 serogroups of *N.meningitidis* have been  
10 identified. Group A is the pathogen most often implicated in epidemic disease in sub-Saharan Africa. Serogroups B and C are responsible for the vast majority of cases in the United States and in most developed countries. Serogroups W135 and Y are responsible for the rest of the cases in the United States and developed countries.

The meningococcal vaccine currently in use is a tetravalent polysaccharide vaccine composed  
15 of serogroups A, C, Y and W135. Meningococcus B remains a problem, however. The polysaccharide approach cannot be used because the menB capsular polysaccharide is a polymer of  $\alpha(2-8)$ -linked *N*-acetyl neuraminic acid that is also present in mammalian tissue. One approach to a menB vaccine uses mixtures of outer membrane proteins (OMPs) To overcome the antigenic variability, multivalent vaccines containing up to nine different porins  
20 have been constructed [eg. Poolman JT (1992) Development of a meningococcal vaccine. *Infect. Agents Dis.* 4:13-28]. Additional proteins to be used in outer membrane vaccines have been the opa and opc proteins, but none of these approaches have been able to overcome the antigenic variability [eg. Ala'Aldeen & Borriello (1996) The meningococcal transferrin-binding proteins 1 and 2 are both surface exposed and generate bactericidal antibodies capable of killing  
25 homologous and heterologous strains. *Vaccine* 14(1):49-53].

Given the propensity for meningococcal disease during non-epidemic periods to be caused by multiple strains or strain variants [Russel *et al.* (1998) *Abstracts of 11th International pathogenic Neisseria conference*, page 281] together with frequent temporal shifts in the predominant strains in a community, it seems that a universal meningococcal B vaccine will  
30 require more than one antigenic species.

**DESCRIPTION OF THE INVENTION**

Neisserial protein and nucleotide sequences are disclosed in the following documents:

- WO 99/24578
- WO 99/36544
- WO 99/57280
- WO 97/28273
- WO 96/29412
- WO 95/03413
- Tettelin *et al.* (2000) *Science* 287:1809-1815

For ease of reference, the sequences disclosed in these documents are referred to in the present application according to the following table:

Document	Original sequence numbering	Sequence numbering in this application
WO 99/24578	SEQ IDs 1-892	SEQ IDs 1-892
WO 99/36544	SEQ IDs 1-90	SEQ IDs 893-982
WO 99/57280	SEQ IDs 1-3020	SEQ IDs 983-4002
WO 97/28273	Figure 4 coding DNA Figure 4 protein Figure 9 coding DNA Figure 13 DNA Figure 13 protein	SEQ ID 4003 SEQ ID 4004 SEQ ID 4005 SEQ ID 4006 SEQ ID 4007
WO 96/29412	SEQ IDs 1-26	SEQ IDs 4008-4033
WO 95/03413	SEQ IDs 1-23	SEQ IDs 4034-4056
Tettelin <i>et al.</i> (2000) <i>Science</i> 287:1809-15	NMB0001-2160 (DNA) NMB0001-2160 (encoded proteins)	SEQ IDs 4057-6216 SEQ IDs 6217-8376

The present invention provides compositions comprising a first biological molecule from a *Neisseria* bacterium and a second biological molecule from a *Neisseria* bacterium. The term "biological molecule" includes proteins and nucleic acids.

The compositions may also comprise further biological molecules, preferably also from *Neisseria*, that is to say the compositions may comprise two or more biological molecules (*eg.* 3, 4, 5, 6, 7, 8 *etc.*), at least two of which are from a *Neisseria* bacterium (*eg.* 3, 4, 5, 6, 7, 8 *etc.*). Such compositions include those comprising (i) two or more different Neisserial proteins.

(ii) two or more different Neisserial nucleic acids, or (iii) mixtures of one or more Neisserial protein and one or more Neisserial nucleic acid.

In one preferred embodiment, the first and second biological molecules are from different *Neisseria* species (eg. one is from *N.meningitidis* and one is from *N.gonorrhoeae*), but they may be from the same species. The biological molecules in the compositions may be from different serogroups or strains of the same species.

The first biological molecule is preferably selected from the group consisting of SEQ IDs 1-8376. More preferably, it is selected from the group consisting of SEQ IDs 1-4002 and/or SEQ IDs 4057-8376. It is preferably a purified or isolated biological molecule.

10 The second biological molecule is preferably selected from the group consisting of SEQ IDs 1-8376. More preferably, it is selected from the group consisting of SEQ IDs 1-4002 and/or SEQ IDs 4057-8376. It is preferably a purified or isolated biological molecule.

One or both of the first and second biological molecules may be a Neisserial biological molecule which is not specifically disclosed herein, and which may not have been identified, discovered, made available to the public or purified before this patent application was filed.

In particular, the invention provides a composition comprising one or more of the following pairs of first and second biological molecules (listed by SEQ ID):

First	Second				
1	2-8376	24	1-23 and 25-8376	48	1-47 and 49-8376
2	1 and 3-8376	25	1-24 and 26-8376	49	1-48 and 50-8376
3	1-2 and 4-8376	26	1-25 and 27-8376	50	1-49 and 51-8376
4	1-3 and 5-8376	27	1-26 and 28-8376	51	1-50 and 52-8376
5	1-4 and 6-8376	28	1-27 and 29-8376	52	1-51 and 53-8376
6	1-5 and 7-8376	29	1-28 and 30-8376	53	1-52 and 54-8376
7	1-6 and 8-8376	30	1-29 and 31-8376	54	1-53 and 55-8376
8	1-7 and 9-8376	31	1-30 and 32-8376	55	1-54 and 56-8376
9	1-8 and 10-8376	32	1-31 and 33-8376	56	1-55 and 57-8376
10	1-9 and 11-8376	33	1-32 and 34-8376	57	1-56 and 58-8376
11	1-10 and 12-8376	34	1-33 and 35-8376	58	1-57 and 59-8376
12	1-11 and 13-8376	35	1-34 and 36-8376	59	1-58 and 60-8376
13	1-12 and 14-8376	36	1-35 and 37-8376	60	1-59 and 61-8376
14	1-13 and 15-8376	37	1-36 and 38-8376	61	1-60 and 62-8376
15	1-14 and 16-8376	38	1-37 and 39-8376	62	1-61 and 63-8376
16	1-15 and 17-8376	39	1-38 and 40-8376	63	1-62 and 64-8376
17	1-16 and 18-8376	40	1-39 and 41-8376	64	1-63 and 65-8376
18	1-17 and 19-8376	41	1-40 and 42-8376	65	1-64 and 66-8376
19	1-18 and 20-8376	42	1-41 and 43-8376	66	1-65 and 67-8376
20	1-19 and 21-8376	43	1-42 and 44-8376	67	1-66 and 68-8376
21	1-20 and 22-8376	44	1-43 and 45-8376	68	1-67 and 69-8376
22	1-21 and 23-8376	45	1-44 and 46-8376	69	1-68 and 70-8376
23	1-22 and 24-8376	46	1-45 and 47-8376	70	1-69 and 71-8376
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8214	1-8213 and 8215-8376
8215	1-8214 and 8216-8376
8216	1-8215 and 8217-8376
8217	1-8216 and 8218-8376
8218	1-8217 and 8219-8376
8219	1-8218 and 8220-8376
8220	1-8219 and 8221-8376
8221	1-8220 and 8222-8376
8222	1-8221 and 8223-8376

8223	1-8222 and 8224-8376
8224	1-8223 and 8225-8376
8225	1-8224 and 8226-8376
8226	1-8225 and 8227-8376
8227	1-8226 and 8228-8376
8228	1-8227 and 8229-8376
8229	1-8228 and 8230-8376
8230	1-8229 and 8231-8376
8231	1-8230 and 8232-8376
8232	1-8231 and 8233-8376
8233	1-8232 and 8234-8376
8234	1-8233 and 8235-8376
8235	1-8234 and 8236-8376
8236	1-8235 and 8237-8376
8237	1-8236 and 8238-8376
8238	1-8237 and 8239-8376
8239	1-8238 and 8240-8376
8240	1-8239 and 8241-8376
8241	1-8240 and 8242-8376
8242	1-8241 and 8243-8376
8243	1-8242 and 8244-8376
8244	1-8243 and 8245-8376
8245	1-8244 and 8246-8376
8246	1-8245 and 8247-8376
8247	1-8246 and 8248-8376
8248	1-8247 and 8249-8376
8249	1-8248 and 8250-8376
8250	1-8249 and 8251-8376
8251	1-8250 and 8252-8376
8252	1-8251 and 8253-8376
8253	1-8252 and 8254-8376
8254	1-8253 and 8255-8376
8255	1-8254 and 8256-8376
8256	1-8255 and 8257-8376
8257	1-8256 and 8258-8376
8258	1-8257 and 8259-8376
8259	1-8258 and 8260-8376
8260	1-8259 and 8261-8376
8261	1-8260 and 8262-8376
8262	1-8261 and 8263-8376
8263	1-8262 and 8264-8376
8264	1-8263 and 8265-8376
8265	1-8264 and 8266-8376
8266	1-8265 and 8267-8376
8267	1-8266 and 8268-8376
8268	1-8267 and 8269-8376
8269	1-8268 and 8270-8376
8270	1-8269 and 8271-8376
8271	1-8270 and 8272-8376
8272	1-8271 and 8273-8376
8273	1-8272 and 8274-8376
8274	1-8273 and 8275-8376
8275	1-8274 and 8276-8376
8276	1-8275 and 8277-8376
8277	1-8276 and 8278-8376
8278	1-8277 and 8279-8376
8279	1-8278 and 8280-8376

8280	1-8279 and 8281-8376	8313	1-8312 and 8314-8376	8346	1-8345 and 8347-8376
8281	1-8280 and 8282-8376	8314	1-8313 and 8315-8376	8347	1-8346 and 8348-8376
8282	1-8281 and 8283-8376	8315	1-8314 and 8316-8376	8348	1-8347 and 8349-8376
8283	1-8282 and 8284-8376	8316	1-8315 and 8317-8376	8349	1-8348 and 8350-8376
8284	1-8283 and 8285-8376	8317	1-8316 and 8318-8376	8350	1-8349 and 8351-8376
8285	1-8284 and 8286-8376	8318	1-8317 and 8319-8376	8351	1-8350 and 8352-8376
8286	1-8285 and 8287-8376	8319	1-8318 and 8320-8376	8352	1-8351 and 8353-8376
8287	1-8286 and 8288-8376	8320	1-8319 and 8321-8376	8353	1-8352 and 8354-8376
8288	1-8287 and 8289-8376	8321	1-8320 and 8322-8376	8354	1-8353 and 8355-8376
8289	1-8288 and 8290-8376	8322	1-8321 and 8323-8376	8355	1-8354 and 8356-8376
8290	1-8289 and 8291-8376	8323	1-8322 and 8324-8376	8356	1-8355 and 8357-8376
8291	1-8290 and 8292-8376	8324	1-8323 and 8325-8376	8357	1-8356 and 8358-8376
8292	1-8291 and 8293-8376	8325	1-8324 and 8326-8376	8358	1-8357 and 8359-8376
8293	1-8292 and 8294-8376	8326	1-8325 and 8327-8376	8359	1-8358 and 8360-8376
8294	1-8293 and 8295-8376	8327	1-8326 and 8328-8376	8360	1-8359 and 8361-8376
8295	1-8294 and 8296-8376	8328	1-8327 and 8329-8376	8361	1-8360 and 8362-8376
8296	1-8295 and 8297-8376	8329	1-8328 and 8330-8376	8362	1-8361 and 8363-8376
8297	1-8296 and 8298-8376	8330	1-8329 and 8331-8376	8363	1-8362 and 8364-8376
8298	1-8297 and 8299-8376	8331	1-8330 and 8332-8376	8364	1-8363 and 8365-8376
8299	1-8298 and 8300-8376	8332	1-8331 and 8333-8376	8365	1-8364 and 8366-8376
8300	1-8299 and 8301-8376	8333	1-8332 and 8334-8376	8366	1-8365 and 8367-8376
8301	1-8300 and 8302-8376	8334	1-8333 and 8335-8376	8367	1-8366 and 8368-8376
8302	1-8301 and 8303-8376	8335	1-8334 and 8336-8376	8368	1-8367 and 8369-8376
8303	1-8302 and 8304-8376	8336	1-8335 and 8337-8376	8369	1-8368 and 8370-8376
8304	1-8303 and 8305-8376	8337	1-8336 and 8338-8376	8370	1-8369 and 8371-8376
8305	1-8304 and 8306-8376	8338	1-8337 and 8339-8376	8371	1-8370 and 8372-8376
8306	1-8305 and 8307-8376	8339	1-8338 and 8340-8376	8372	1-8371 and 8373-8376
8307	1-8306 and 8308-8376	8340	1-8339 and 8341-8376	8373	1-8372 and 8374-8376
8308	1-8307 and 8309-8376	8341	1-8340 and 8342-8376	8374	1-8373 and 8375-8376
8309	1-8308 and 8310-8376	8342	1-8341 and 8343-8376	8375	1-8374 and 8376
8310	1-8309 and 8311-8376	8343	1-8342 and 8344-8376	8376	1-8375
8311	1-8310 and 8312-8376	8344	1-8343 and 8345-8376		
8312	1-8311 and 8313-8376	8345	1-8344 and 8346-8376		

Thus the invention includes each of the 35074500 possible pairs of SEQ IDs 1-8376 (1&2, 1&3, 1&4, 1&5 ... 1&8375, 1&8376, 2&3, 2&4, 2&5 ... 2&8375, 2&8376, 3&4 ... 1000&1001, 1000&1002 ... 1000&8376 ... 8374&8375, 8374&8376, 8375&8376) although, for reasons of space, these are not listed in full here.

Details as to how the molecules which make up the SEQ IDs 1-4056 can be produced and used can be found from the relevant international applications and these details need not be repeated here. Similar principles apply to SEQ IDs 4057-8376.

- 10 SEQ IDs 1-8376 in the compositions of the invention may be supplemented or substituted with molecules comprising sequences homologous (*ie.* having sequence identity) to SEQ IDs 1-8376. Depending on the particular sequence, the degree of identity is preferably greater than 50% (*eg.* 65%, 80%, 90%, or more), and include mutants and allelic variants. Sequence identity between the proteins is preferably determined by the Smith-Waterman homology search

algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty*=12 and *gap extension penalty*=1.

SEQ IDs 1-8376 in the compositions of the invention may be supplemented or substituted with molecules comprising fragments of SEQ IDs 1-8376. Such fragments should comprise at least *n* consecutive monomers from the molecules and, depending on the particular sequence, *n* is either (i) 7 or more for protein molecules (eg. 8, 10, 12, 14, 16, 18, 20 or more), preferably such that the fragment comprises an epitope from the sequence, or (ii) 10 or more for nucleic acid molecules (eg 12, 14, 15, 18, 20, 25, 30, 35, 40 or more).

Where the composition includes a protein that exists in different nascent and mature forms, the mature form of the protein is preferably used. For example, the mature form of the NspA protein (SEQ IDs 4008-4033; WO96/29412; Figure 29) lacking the signal peptide may be used.

In the case of protein molecules, SEQ IDs 1-8376 in the compositions of the invention may be supplemented or substituted with an antibody that binds to the protein. This antibody may be monoclonal or polyclonal.

In the case of nucleic acid molecules, SEQ IDs 1-8376 in the compositions of the invention may be supplemented or substituted with nucleic acid which can hybridise to the *Neisseria* nucleic acid, preferably under "high stringency" conditions (eg. 65°C in a 0.1xSSC, 0.5% SDS solution).

It will be appreciated that any nucleic acid in the compositions can take various forms (eg. single stranded, double stranded, vectors, probes *etc.*). In addition, the term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) *etc.*

In certain embodiments, the composition comprises molecules from different *Neisseria* species, such as one or more *N.meningitidis* molecule and one or more *N.gonorrhoeae* molecule. In some embodiments, the composition may comprise molecules from different serogroups and/or strains of the same species, such as strains A and B of *N.meningitidis*. Further embodiments comprise mixtures of one or more *N.meningitidis* molecules from different strains and also one or more *N.gonorrhoeae* molecules.

Many proteins are relatively conserved between different species, serogroups and strains of *N.meningitidis* and *N.gonorrhoeae* (eg. SEQ IDs 52, 54, 58). PCT/IB00/00642 includes a more

detailed experimental analysis of conserved regions in these proteins. To ensure maximum cross-strain recognition and reactivity, regions of proteins that are conserved between different Neisserial species, serogroups and strains can be used in the compositions of the present invention. The invention therefore provides proteins which comprise stretches of amino acid  
5 sequence that are shared across the majority of *Neisseria*, particularly *N.meningitidis* and *N.gonorrhoeae*. Preferably, therefore, the composition comprises a protein comprising a fragment of a Neisserial protein (preferably a protein from SEQ IDs 1-8376, or more preferably SEQ IDs 1-4002), wherein said fragment consists of  $n$  consecutive conserved amino acids. Depending on the particular protein,  $n$  is 7 or more (eg. 8, 10, 12, 14, 16, 18, 20 or more). The  
10 fragment preferably comprises an antigenic or immunogenic region of the Neisserial protein. A "conserved" amino acid is one that is present in a particular Neisserial protein in at least  $x\%$  of *Neisseria* (or, preferably, in at least  $x\%$  of combined *N.meningitidis* and *N.gonorrhoeae* strains). The value of  $x$  may be 50% or more eg. 66%, 75%, 80%, 90%, 95% or even 100% (ie. the amino acid is found in the protein in question in all *Neisseria*). In order to determine  
15 whether an amino acid is "conserved" in a particular Neisserial protein, it is necessary to compare that amino acid residue in the sequences of the protein in question from a plurality of different *Neisseria* (a "reference population"). Suitable definitions of "reference populations" can be found in PCT/IB00/00642. Amino acid sequences of different *Neissieriae* can easily be compared using computers. This will typically involve the alignment of a number of sequences  
20 using an algorithm such as CLUSTAL [Thompson *et al.* (1994) *Nucleic Acids Res* 22:4673-4680; *Trends Biochem Sci* (1998) 23:403-405] or, preferably, PILEUP [part of the GCG Wisconsin package, preferably version 9.0]. Conserved amino acids are readily apparent in a multiple sequence alignment – at the amino acid position in question a majority of the aligned sequences will contain a particular amino acid. Conserved amino acids can be made more  
25 visually apparent by using a program such as BOXSHADE [available, for instance, at the NIH on-line], PRETTYBOX [GCG Wisconsin, version 10] or JALVIEW [available on-line at EBI].

Specific compositions according to the invention therefore include those comprising:

- two or more biological molecules selected from SEQ IDs 1-4002;
- one or more biological molecules selected from SEQ IDs 1-4002 combined with one or  
30 more biological molecules selected from SEQ IDs 4003-8376;
- one or more biological molecules selected from SEQ IDs 1-4002 combined with the NspA protein (as disclosed in WO96/29412: see also Figure 29 herein), preferably in mature form:



- one or more biological molecules selected from SEQ IDs 1-8376 (preferably SEQ IDs 1-4002) combined with transferrin binding protein A (TbpA) and/or B (TbpB), such as the TbpA and TbpB disclosed in WO00/25811 (or immunogenic fragments thereof).
- one or more fragments of proteins selected from SEQ IDs 1-4002, with the fragment preferably comprising a stretch of conserved amino acids;
- a combination of different proteins, wherein the combination as a whole includes one or more proteins that is recognised by each strain in a reference population, although each individual protein in the combination may not itself be recognised by each strain in the reference population *ie.* each member of a reference population recognises at least one protein in the combination.

The invention also provides the compositions of the invention for use as medicaments (*eg.* as immunogenic compositions or vaccines) or as diagnostic reagents. It also provides the use of the compositions in the manufacture of: (i) a medicament for treating or preventing infection due to Neisserial bacteria; (ii) a diagnostic reagent for detecting the presence of Neisserial bacteria or of antibodies raised against Neisserial bacteria; and/or (iii) a reagent which can raise antibodies against Neisserial bacteria.

The invention also provides a method of treating a patient, comprising administering to the patient a therapeutically effective amount of a composition according to the invention.

The invention further provides a process for producing a composition according to the invention, comprising the step of bringing one or more of SEQ IDs 1-8376 into combination with one or more other of SEQ IDs 1-8376.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows SDS-PAGE results for the expression of ORFs 6, 7, 13, 65-1, 72, 73-1, 105-1, 137-1, 143-1 and 147-1. The left-hand lanes show molecular weight markers (set M1).

**Figure 2** shows (A) SDS-PAGE results for ORF9 (B) the position of the *N.meningitidis* immunoreactive band in a Western blot against a *N.meningitidis* outer membrane vesicle preparation (C) FACS analysis.

**Figure 3** shows SDS-PAGE results for the expression of ORFs 2-1, 5-1, 22-1, 132-1 and 4. The left-hand lanes show molecular weight markers (set M1).

**Figures 4 to 28** show a hydrophilicity plot (upper), antigenic index (middle), and AMPHI regions (lower) for ORFs 2, 5, 6, 7, 9, 13a, 15, 22, 23, 27, 28, 32, 65, 72, 73, 76, 79, 89, 105, 106-1, 132, 137, 138, 143 and 147.

**Figure 29** shows sequence variability of NspA from various strains of meningococcus B. These sequences may be used as alternatives to the NspA of WO 96/29412 (SEQ IDs 4008-4033).

**Figure 30** shows the binding of polyclonal anti-rNspA by indirect fluorescence flow cytometry to encapsulated and non-encapsulated menB strains. **Figure 31** shows similar data for encapsulated strains 8047, CU385 & M986 (31A) and non-encapsulated strains BZ232, MC58, NG3/88 & NGB165 (31B).

**Figure 32** shows a model of NspA secondary structure.

**Figure 33** shows FACS analysis of non-encapsulated strain M7 (37A) and ethanol-treated (to disrupt the capsule) strains 2996, N44776, MC58, 1000, BZ232, BZ133, NG6/88, BZ198, NG3/88, 297-0, BZ147 and BZ169 (37B) using a tetravalent mixture.

**Figure 34** shows FACS analysis of M7 strain using a pentavalent mixture at 1:400 (34A), 1:200 (34B) & 1:100 (34C) dilutions.

## EXAMPLES

### Example 1 – Expression and purification experiments

ORFs 6, 7, 13, 65-1, 72, 73-1, 105-1, 137-1, 143-1 and 147-1 disclosed in WO99/24578 were expressed in *E.coli* and purified, as set out in the following table:

ORF	His-fusion	GST-fusion	Purification	MW (kDa)
6		+	GST-fusion	23
7	+	+	GST-fusion	28
13		+	GST-fusion	10
65-1		+	GST-fusion	32
72	+		His-fusion	13.5
73-1		+	GST-fusion	13
105-1	+	+	His-fusion	32
137-1		+	GST-fusion	31
143-1	+	+	His-fusion	23.5
147-1	–	+	His-fusion	32

NOTE: ORF73-1 was expressed as a fragment (amino acids 41-161)

The protocols used to express these ten ORFs were essentially the same as those described in WO99/24578, using pGEN and pET vectors. Examples of the PCR primers used to amplify the ORFs are in the following table:

ORF	Primer	Sequence	Restriction sites
ORF65-1	Forward	CGCGGATCCCATATG-TTTATGAACAAATTTTCC	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-TTTGCTTTCGATAGAACGG	
ORF73-1	Forward	CGCGGATCCCATATG-GCCGGCGTGCTGATG	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-TTCATCTTTTTCATGTTTCG	
ORF105-1	Forward	CGCGGATCCCATATG-CCGACCGTCCGTT	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-TAAACGAATGCCGTCCAG	
ORF143-1	Forward	CGCGGATCCCATATG-GAATCAACACTTTTCAC	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-CACGCGGTTGCTGTAAC	
ORF147-1	Forward	CCGGAATTCTACATATG-TTTCAGAAACATTTGCAG	EcoRI-NdeI XhoI
	Reverse	CCCGCTCGAG-TTTGTTTTTCCAAGACAGAG	

5 SDS-PAGE results for the ten expressed ORFs are shown in Figure 1.

The ORF7-His fusion was used to immunise mice. The sera were used in an ELISA assay, essentially as described in WO99/24578, and gave positive results.

The following proteins were also expressed and purified (results not shown):

ORF	His-fusion expression	GST-fusion expression	Expressed as a fragment	Fragment amino acid positions
orf1 (s)	-	-	-	43-1087
orf1 ( $\beta$ )	n.d.	-	-	1051-1457
orf35-1	-	-	-	
orf41	n.d.	n.d.	-	25-619
orf46	-	-	-	25-433
orf61	n.d.	n.d.	-	1-575

orf83-1	-	-	-	15-313
orf100-1	n.d.	-	-	21-376
orf114-1	n.d.	-	-	1-1423
orf124-1	n.d.	n.d.	-	
orf131-1	n.d.	-	-	22-135

The following PCR primers were used to amplify these ORFs:

ORF	Primer	Sequence	Restriction sites
ORF1 (s)	Forward	CGCGGATCCGCTAGC-GGACACACTTATTTCCG	BamHI-NheI XhoI
	Reverse	CCCGCTCGAG-CAGCGCGTCAAGGCTT	
ORF1 (β)	Forward	CGCGGATCCGCTAGC-GAGTTCCGCCTGCATAA	BamHI-NheI XhoI
	Reverse	CCCGCTCGAG-CCAGCGGTAGCCTAATT	
ORF35-1	Forward	CGCGGATCCGCTAGC-AGGGGCGACGACGTG	BamHI-NheI XhoI
	Reverse	CCCGCTCGAG-AAACAGCCATTTGAGCGA	
ORF41	Forward	CGCGGATCCCATATG-TATTTGAAACAGCTCCAAG	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-TTCTGGGTGAATGTTA	
ORF46	Forward	CGCGGATCCCATATG-TCAGATTTGGCAAACGATC	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-CGTATCATATTTCACTGTC	
ORF61	Forward	CGCGGATCCGCTAGC-ACGGTTTTGAAGCTTTTCGC	BamHI-NheI XhoI
	Reverse	CCCGCTCGAG-AATGACGAGGTTGTCCG	
ORF83-1	Forward	CGCGGATCCCATATG-TGCGGCACACTGACCG	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-GCCGCCTTTGCGGC	
ORF100-1	Forward	CGCGGATCCCATATG-TCGGGCATTTACACCGG	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-TTCGTGAAAACCTTTGC	
ORF114-1	Forward	CGCGGATCCCATATG-AATAAAGGTTTACATCGCAT	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-AATCGCTGCACCGGCT	
ORF124-1	Forward	CGCGGATCCCATATG-ACTGCCTTTTCGACA	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-GCGTGAAGCGTCAGGA	

<b>ORF131-1</b>	Forward	CGCGGATCCCATATG-TGCCGGCTGGCGGG	BamHI-NdeI XhoI
	Reverse	CCCGCTCGAG-CCAGCGGACGCGTTC	

Each of these ORFs can be combined with one or more of SEQ IDs 1-8376.

### Example 2 – ORF9 expression and purification

ORF9 disclosed in WO99/24578 was cloned in the pET vector and expressed in *E.coli*. The purified ORF-His fusion protein was analysed by SDS-PAGE, as shown in Figure 2A. Mice were immunised with the purified ORF9-His and sera were used for Western blot analysis (Figure 2B), FACS analysis (Figure 2C), and ELISA assay. The protocols used were essentially the same as those set out in WO99/24578.

The results confirm that ORF9 is a surface-exposed protein. ORF9 is suitable for combining with one or more other of SEQ IDs 1-8376.

### 10 Example 3 – Further expression experiments

Further expression and purification experiments were carried out in *E.coli* for ORFs 2-1, 5-1, 22-1 and 132-1 disclosed in WO99/24578, as set out in the following table:

ORF	His-fusion	GST-fusion	Purification	MW (kDa)
2-1		+	GST-fusion	26
5-1	-	+	GST-fusion	33
22-1	+	+	His-fusion	49
132-1	-	+	GST-fusion	48

The protocols used to express these four ORFs were essentially the same as those described in WO99/24578, using pGENX and pET vectors. Examples of the PCR primers used to amplify the ORFs are in the following table:

ORF	Primer	Sequence	Restriction sites
<b>ORF5-1</b>	Forward	CGCGGATCCGCTAGC-GACGGCGCACAACCGA	BamHI-NheI

ORF22-1	Reverse	CCCGCTCGAG-TGTTTGGCGGATGGGG	XhoI
	Forward	CGCGGATCCCATATG-ATTAAAATCAAAAAAGGTC	BamHI-NdeI
	Reverse	CCCGCTCGAG-GCCTTCCTTCTCAATGG	XhoI
ORF132-1	Forward	CGCGGATCCCATATG-AAAGAAGCGGGGTTTGAA	BamHI-NdeI
	Reverse	CCCGCTCGAG-TCTCAAAGCTTCCAGCAG	XhoI

SDS-PAGE results for the four expressed ORFs are shown in Figure 3.

Each of these ORFs can be combined with one or more of SEQ IDs 1-8376.

#### Example 4 – ORF4 lipoprotein expression and purification

ORF4 is disclosed in WO99/24578 as containing a lipopeptide signal sequence (LPSS). The full length ORF was amplified using the following PCR primers:

orf4-L for CGCGGATCCCATATGAAAACCTTCTTCAAAACC

orf4-L rev CCCGCTCGAGTTATTTGGCTGCGCCTTC

The amplified DNA fragment was cloned into the vector pET21b+ for expression as a C-terminus His-tagged fusion. A log phase culture of *E.coli* containing pET21b+orf4-LPSS was induced with 1.0 mM IPTG for 3 h at 30°C, collected by centrifugation for 10 min at 8000g, and resuspended in PBS. The suspension was sonicated on ice and Triton X-114 added to a final concentration of 0.6% (v/v). The material was incubated on ice for 20 minutes then warmed to 37°C until phase separation occurred (indicated by a high degree of cloudiness). After centrifugation for 10 min at 10,000g at 20°C the upper aqueous phase was discarded, and the lower detergent phase collected without disrupting the bacterial pellet. To the detergent phase was added 13 volumes of 20 mM Histidine, 2 mM EDTA, 30 mM NaCl (pH 5.8). This was centrifuged for 10 min at 4°C and the supernatant combined batchwise at 4°C for 30 min, with Q Sepharose Fast Flow resin (Pharmacia). The mixture was centrifuged, the supernatant retained and the resin washed with 20mM Histidine, 2mM EDTA, 30 mM NaCl (pH 5.8), Triton X-100 0.3% (v/v) and eluted with 1M NaCl in the same buffer. The majority of Orf4 lipoprotein was found in the supernatant obtained after binding. Final purification was accomplished by chromatography on Hi-Trap™ Q (Pharmacia). The binding-supernatant was adjusted to pH 7.0 by the addition of 0.1M HCl and applied to a Hi-Trap™ Q column

equilibrated with 50mM Tris-HCl (pH 7.0), 2mM EDTA, 0.3% Triton X-100, 10mM NaCl. The column was washed with 5.0 ml of the equilibration buffer and a NaCl gradient from 10mM to 1M was applied. Two electrophoretically distinct forms of the protein eluted. One in the wash and the other in the NaCl gradient between 150mM and 300mM NaCl. The protein  
 5 obtained in the wash was used for immunization of mice. This form of the protein probably represents the fully processed, lipidated molecule.

The 31kDa purified lipoprotein can be seen in Figure 3. ORF4 is suitable for combining with one or more other of SEQ IDs 1-8376.

#### Example 5 – Computer prediction

10 Computer analysis of ORFs 2, 5, 6a, 7, 9, 13a, 15, 22, 23, 27, 28, 32, 65, 72, 73, 76, 79, 89, 105, 106-1, 132, 137, 138, 143 and 147 (as disclosed in WO99/24578) was performed. Figures 4 to 28 show, for each of these ORFs, a hydrophilicity plot (upper), an antigenic index plot (middle), and an AMPHI analysis (lower). The AMPHI program has been used to predict T-cell epitopes [Gao *et al.* (1989) *J. Immunol.* 143:3007; Roberts *et al.* (1996) *AIDS Res Hum Retrovir*  
 15 12:593; Quakyi *et al.* (1992) *Scand J Immunol* suppl.11:9) and is available in the Protean package of DNASTAR, Inc. (1228 South Park Street, Madison, Wisconsin 53715 USA).

Each of these ORFs can be combined with one or more other of SEQ IDs 1-8376.

#### Example 6 – Tetravalent mixture

A mixture of proteins 919 (WO99/57280), 225 (WO99/57280), ORF4 (WO99/24578, example  
 20 26) and ORF40 (WO99/36544, example 1) was produced and assessed by ELISA and FACS. The ELISA titres against 13 test strains were as follows:

<b>Strain</b>	M7 conF	2996	MC58	BZ133	BZ232	H44/76	1000
<b>Titre</b>	38176	7892	17216	41488	17945	83990	6575
<b>Strain</b>	BZ198	NG6/88	BZ169	BZ147	NG3/88	297-0	
<b>Titre</b>	3329	59275	-	-	12877	25640	

The FACS results are shown in Figure 33. It is evident that the tetravalent mixture gives excellent results, regardless of the particular menB strain used. In addition, antisera raised  
 25 against the mixture in strain 2996 is bactericidal at up to 1:2048 dilution.



**Example 7 – Pentavalent mixture**

A mixture of proteins ORF4-L (the lipidated protein – see example 4 above), ORF37 (WO99/24578, example 1), ORF40 (WO99/36544, example 1), 502 (WO99/57280, pages 687-690) and 8 (WO99/57280, pages 165-167) was produced. The ELISA titres against 13 test strains were as follows:

<b>Strain</b>	M7 conF	2996	MC58	BZ133	BZ232	H44/76	1000
<b>Titre</b>	-	-	25428	-	58300	>109350	>109350
<b>Strain</b>	BZ198	NG6/88	BZ169	BZ147	NG3/88	297-0	
<b>Titre</b>	10999	109532	42888	28324	104212	33996	

The FACS results are shown in Figure 34. It is evident that the pentavalent mixture gives excellent results, regardless of the particular menB strain used. In addition, antisera raised against the mixture in strain 2996 is bacteriostatic.

**10 Example 8 – trivalent mixture**

Proteins ORF1 (*e.g.* example 77 of WO99/24578; see also WO99/55873), '287' (*e.g.* Figure 21 of WO99/57280; also SEQ IDs 3103-3108 therein) and '919' (*e.g.* WO99/57280 Figure 23 and SEQ IDs 3069-3074 therein) were combined and adjuvanted with Al(OH)<sub>3</sub>. The proteins were from the 2996 strain of MenB.

15 This mixture was also combined with a MenC polysaccharide conjugate antigen [*e.g.* Costantino *et al.* (1992) *Vaccine* 10:691-698]. OMVs were used as controls.

The mixture was used in a bactericidal assay against the homologous strain and also heterologous MenB strains. Titres were as follows:

	<b>2996</b>	<b>BZ133</b>	<b>BZ232</b>	<b>1000</b>	<b>MC58</b>	<b>NGH38</b>
<b>Trivalent</b>	2048	2048	4	<4	64	4
<b>+ MenC</b>	2048	>32000	4	128	1024	128
<b>Control</b>	32765	4096	8192	16384	16384	8192

**Example 9 – Proteins 287, 919 and 953**

Proteins 287, 919 and 953 are disclosed in WO99/57280. These proteins from *N.meningitidis* serogroup B strain 2996 were expressed and tested in a bactericidal assay against strain 2996, alone and in combinations. OMVs from 2996 were used as a positive control.

Antigen	287	919	953	Control
<b>Titre</b>	8192	2048	128	65536
<b>Combination</b>	<b>287+919</b>	<b>287+953</b>	<b>919+953</b>	<b>287+919+953</b>
<b>Titre</b>	32000	8192	8192	8192

5

Figure 35 shows FACS data for the individual antigens and for the four combinations.

It is evident that the antigen mixtures are more effective than the antigens in isolation and, in particular, that combinations of 919+953 give surprisingly good results.

The individual antigens from 2996 and combinations were also tested against different serogroup A, B & C strains (*i.e.* heterologous challenge). Bactericidal titres were as follows:

10

Antigen	Serogroup B (MenB) strains					MenA	MenC
	2996	BZ133	BZ232	MC58	NGH38	F6124	C11
<b>287</b>	8192	>4096	256	1024	2048	1024	2048
<b>919</b>	2048	-	1024	-	-	-	-
<b>953</b>	128	-	-	-	-	-	-
<b>287+919</b>	32000	>4096	512	512	1024	512	>2048
<b>287+953</b>	8192	>4096	1024	512	2048	2048	>2048
<b>919+953</b>	8192	-	8192	-	-	-	-
<b>Trivalent</b>	8192	>2048	256	-	1024	>2048	>2048
<b>Control</b>	65536	-	8192	2048	-	2048	32768

It is apparent that the antigen mixtures are useful in conferring cross-strain activity.

In a second set of experiments, titres for the individual antigens were as follows:

Antigen	Serogroup B (MenB) strains					MenA	MenC
	2996	BZ133	BZ232	MC58	NGH38	F6124	C11
<b>287</b>	16000	2048	16	512	>2048	64	1024
<b>919</b>	16000	-	2048	-	-	-	-
<b>953</b>	2048	-	16	-	-	-	-

The three proteins used in this example were expressed and used in the following forms:

- (1) Protein 287 was expressed in *E.coli* as a GST fusion;
- (2) Protein 919 was expressed in *E.coli* without its leader peptide, without its mature N-terminal cysteine, and without any fusion partners ("919-untag"); and
- (3) Protein 953 was expressed using a histidine tag.
- 5 Three immunisations were administered with Freund's adjuvants – the first included CFA, and the final two included IFA.

### Example 10 – further polyvalent combinations

Further combinations of antigens were tested in CD1 mice:

Antigens *	Adjuvant	FACS	ELISA	Bactericidal activity
919-his+Orf4-his+ 225-his+Orf40-his	Freund	+++	+	8192
Orf4-L+Orf37-GST+Orf40-his+502-his+8-his	Freund	+++	+	Bacteriostatic
919-untag+791-his+792-his	Freund	+++	+	4096
919-untag+287-GST+953-his	Freund	+++	+	8192
919-untag+287-GST	Freund	+++	+	32000
287-GST+953-his	Freund	+++	+	8192
919-untag+953-his	Freund	+++	+	8192
919-untag+Orf1-his+287-GST	Al(OH) <sub>3</sub>	+++	+	2048
919-untag+Orf1-his+287-GST+MenC glycoconj.	Al(OH) <sub>3</sub>	+++	+	2048
Orf-46.1-his+ 287-GST	Al(OH) <sub>3</sub>	n.d.	+	128

\* : "his" indicates expression and immunisation with a histidine-tagged protein;

"ORF4-L" is the lipidated form of ORF4;

"GST" indicates expression and immunisation with a GST fusion protein;

"919-untag" is as defined in Example 9;

"MenC glycoconj" is the MenC glycoconjugate described in Example 8.

Further combinations of antigens were tested in guinea pigs:

Antigens	Adjuvant	FACS	ELISA	Bactericidal activity
919-his+287-GST+953-his+Orf46.1-his	Freund	+	+	4096
919-untag + 287-GST + 953-his	Freund	+	+	4096
287-GST + 953-his	Al(OH) <sub>3</sub>	n.d.	+	256

Evidently the combinations give excellent immunological results.

### Example 11 – NspA combinations

NspA protein is disclosed in WO96/29412, and is represented herein as SEQ IDs 4008-4033. The academic literature disclosure of this protein [Martin *et al.* (1997) *J. Exp. Med.* 185 1173-  
5 1183] reported the protein to be highly conserved between *Neisseria* strains (99% cross-reactivity of anti-NspA antibodies with 250 meningococcal A, B & C strains) and also efficient protection against deadly challenge with live bacteria. There have also been reports that NspA adsorbed on alum elicits serum meningococcal bactericidal antibody responses in rabbits and monkeys [Martin *et al.* (1998) *Abstracts of 11th International pathogenic Neisseria conference*,  
10 page 198]. On the basis of these data, rNspA (recombinant NspA) is being developed as a vaccine for the prevention of meningococcal disease caused by all serogroups.

Despite sequence conservation, however, it has surprisingly been discovered that rNspA cell surface epitopes are detected on only 65% of the serogroup B strains tested below, and susceptibility to anti-NspA bactericidal activity is also less than that reported by Martin *et al.*  
15 These results contrast with Martin *et al.*, and suggest that a rNspA-based meningococcal B vaccines will need to be supplemented with additional antigens in order to be effective.

The *N.meningitidis* strains tested in this example were isolated from patients residing in different countries over a period of more than 30 years (see table on page 72). These strains were selected to be representative of widely divergent 'clonal' groups, as defined by multilocus  
20 isoenzyme typing [Seiler *et al.* (1996) *Mol. Microbiol.* 19:841-856] and/or multilocus sequence typing [Maiden *et al.* (1998) *PNAS USA* 95:3140-45]. Strain M7, which is derived from strain NMB, contains a transposon insertion that blocks capsular polysaccharide biosynthesis [Stephens *et al.* (1991) *Infect. Immun.* 59:4097-4102], but all the other strains are encapsulated.

Based on the nucleotide sequence in Martin *et al.* (1997), PCR primers were designed and the  
25 *NspA* gene from strain 8047 was amplified. The sequence, including the promoter region, was cloned into pSK+ plasmid (rNspA). A plasmid pTrc.NspA.1 encoding a protein in which a portion of the signal sequence has been replaced with a poly-histidine tag was also used. Both plasmids were expressed in *E.coli* strain BL21(DE3) and the proteins were purified. In *E.coli*, rNspA is secreted, rather than being associated with the outer membrane. The protein was  
30 partially purified from the culture medium by precipitation with 55% w/v ammonium sulphate, and had an apparent MW of 18.6kDa, confirmed by Western Blot.

The two forms of NspA (rNspA and denature His-tag NspA) were injected into 6-week old female CD-1 mice to raise antisera. The ability of these to bind to the surface of *N.meningitidis* strain B was determined using flow cytometric detection of indirect fluorescence assay [Granoff *et al.* (1998) *J.Immunol.* 160:5028-36]. The results for strains NMB and M7 (an acapsulated mutant of NMB) are shown in Figure 30. As expected, anti-group B polysaccharide mAb SEAM-3 [Granoff *et al.*] only binds to the encapsulated strain, whereas the positive anti-P1.2 (PorA) control mAb binds to both strains. The antisera raised against rNspA is able to bind both strains. Antisera against the His-tag NspA gave negative results, however. These antisera were also negative for strains 8047, CU385 and M986 (Figure 31A), but by Western Blot these antisera gave positive results.

These data suggest that antibodies prepared using His-tag NspA recognise epitopes that are present in denatured NspA, but not native NspA as found on the cell-surface *in vivo*. In contrast, antibodies prepared against rNspA seem to recognise conformational NspA epitopes.

The flow cytometric assay was applied to the strains shown in the table on page 72. Figure 31A shows that murine antibodies raised against rNspA bind to the surface of strain 8047 (the strain from which the *nspA* gene was cloned) and strain CU385, but not to M986. Figure 31B shows similar negative results for strains BZ232, MC58, NG3/88 and NGP165. In all of these negative cases, however, the anticapsular mAb control was positive.

The table on page 72 summarises the flow cytometry results. Although NspA is reported to be accessible at the surface of all intact *N.meningitidis* strains tested [Martin *et al.* (1997) *J. Exp. Med* 185 1173-1183; Plante *et al.* (1999) *Infect. Immun.* 67:2855-61], only 11 of the 17 test strains (65%) reacted with the anti-rNspA sera. There was no apparent relationship between cell-surface expression in a given strain and classification (by serotype, subtype, or electrophoretic type), or with year or country of isolation.

In an attempt to explain the differences in reactivity with the anti-rNspA sera, the *nspA* genes from five of the six negative strains (BX232, NG3/88, NGP165, M136 & M986) and from three of the positive strains (8047, CU385 & NG6/88) were sequenced. The sequence for the sixth negative strain (MC58) was already available from the complete genome sequence.

The *nspA* sequences for all ten strains were highly conserved, varying at most by 5 nucleotides from the prototype sequence of Martin *et al.* The most variant protein had only 3 amino acid differences (see Figure 29). With one exception, all of the amino acid variants involved the

same respective residues in discrete segments of the protein. These include the signal peptide, which is not present in the mature protein, and two short segments in the 50 C-terminal residues. These differences do not explain the antisera results, as there are examples of identical variant sequences in strains that were positive and those that were negative (compare M136 & 8047; NGP165 & NG6/88; MC58 & CU385).

As neither lack of the gene nor polymorphism explained the antiserum results, the amount of NspA protein in the outer membranes of five strains (8047, CU385 & NG6/88 – all positive for anti-rNspA; M986 & M136 – both negative) were tested. Bacterial cell pellets were extracted with lauryl sarcosinate, and the insoluble outer membrane fractions were analysed. An 18.6kDa band was seen for all five strains, and this was cross-reactive with anti-His-tag-NspA by Western Blot. Thus strain differences in *nspA* expression also failed to explain the results.

The ability of anti-rNspA to bind to the bacterial cell surface could be influenced by the amount of polysaccharide capsule present. The quantity of capsular polysaccharide produced by the 17 test strains was therefore assessed by inhibition ELISA.

Extracts of capsular polysaccharide were prepared based on a method described by Corn *et al.* [*J.Infect.Dis.* (1993) 167:356-64]. Individual bacterial clones were grown to an OD<sub>620</sub> 0.5-0.7 in 7 mL of Mueller-Hinton broth. Bacteria were collected by centrifugation at 5000g for 15 min, washed in 0.6mL of 10 mM Hepes, pH 8.0, and then resuspended in 0.6 mL of the same buffer containing 10 mM EDTA and incubated at 37°C for 1 hr. The cells were pelleted at 10,000g for 1 minute and the relative amount of meningococcal B polysaccharide antigen released into the supernatant was determined by an inhibition ELISA, performed as described by Azmi *et al.* [*Infect. Immun.* (1995) 63:1906-13]. The solid phase antigen in the ELISA was meningococcal B polysaccharide-ADH-biotin absorbed to avidin-coated microtiter plates [Granoff *et al.*]. The meningococcal B polysaccharide-reactive human paraprotein LIP [Azmi *et al.*] was used as the primary antibody (0.2 µg/ml). In the absence of inhibitor, this concentration of antibody was sufficient to given an OD of ~0.7 to 1.0 after 30 minutes incubation with substrate [Azmi *et al.*]. The titre of polysaccharide released into the supernatant was measured by determining the dilution of supernatant that resulted in 50% inhibition of antibody binding. Controls in this assay included an EDTA extract prepared from the strain M7, which does not produce any capsular polysaccharide, and purified meningococcal B polysaccharide. To ensure that all of the capsular polysaccharide was released by the EDTA treatment, the same inhibition ELISA was performed using the cell pellet resuspended in the same buffer and volume as the capsule

extract. The observable inhibitory activity from the cell pellet was between 0 and 10% of the activity observed in the capsule extracts with the latter, higher percentage coming from cell pellets of strains that produce the largest amounts of capsule.

The results for each strain are shown in the table on page 72. On average, the six negative anti-rNspA strains produced three-fold more capsular polysaccharide than the eleven positive strains (respective reciprocal geometric mean dilutions of 676 vs. 224,  $p < 0.05$ ). This may explain the results obtained with the antiserum – conceivably, the presence of larger amounts of capsule could interfere with the ability of the anti-rNspA antibody to bind to NspA epitopes which, in strains with lower amounts of capsule, are accessible.

10 The complement-dependent bactericidal activity of the anti-rNspA antisera were tested using an assay similar to that described by Mandrell *et al.* [*J. Infect. Dis.* (1995) 172:1279-89]. The complement source was human serum from a healthy adult with no detectable anti-capsular antibody to group B polysaccharide and no intrinsic bactericidal activity against the test strain. Serum bactericidal titres were defined as the serum dilution resulting in a 50% decrease in  
15 CFU/ml after 60 minutes incubation of bacteria in the reaction mixture, compared to the control CFU/ml at time zero.

Typically, bacteria incubated with a negative control antibody showed a 150-200% increase in CFU/ml during the 60 minutes of incubation. The positive control antibody [anti-capsular IgG2a mAb SEAM 12, Granoff *et al.*] showed complement-mediated bactericidal activity  
20 against all 17 strains. In contrast, the six strains that were negative for anti-rNspA antisera binding by flow assay were resistant, showing no bactericidal or bacteriostatic effects. Ten of the other eleven positive strains were either killed by complement and the antisera (SWZ107, J351, CU385, NG6/88, BZ198, H44/76, NMB & 8047) or were inhibited (H355 & S3446); strain 1000, however, was not affected.

25 The ability of the anti-rNspA antisera to confer passive protection against meningococcal B bacteremia was tested in infant rats using a method adapted from Saukkonen [*J. Infect. Dis.* (1988) 158:209-212]. Briefly, 6-7 day old rats were randomly distributed to nursing mothers. Groups of 5-6 animals were challenged IP with 100µl of approximately 5000 CFU of *N.meningitidis* group B bacteria. One strain negative for NspA surface epitopes (M986) and one  
30 positive strain (8047) were tested, each of which having been passaged three times in infant rats. Immediately before administration, the bacterial suspension was mixed with different dilutions of test or control antibody (positive control: anticapsular mAb; negative control:



anti-*E.coli*). 18 hours after challenge, blood specimens were obtained from the heart. Aliquots were plated onto chocolate agar, and CFU/ml was determined after overnight incubation at 37°C in 5% CO<sub>2</sub>.

The protective activities of the various co-administered antibodies were as follows:

Antibody treatment	Dose per rat or serum dilution	Strain	Blood culture		
			Positive/ total	CFU/ml (mean $\times 10^{-3}$ )	CFU/ml (% of control)
Anticapsular mAb	2 $\mu$ g	M986	0/6	<1	<1
Anti-rNspA	1:5	M986	6/6	44 <sup>a</sup>	45
Anti-rNspA	1:25	M986	6/6	93 <sup>a</sup>	95
Anti- <i>E.coli</i> control	1:5	M986	6/6	98 <sup>a</sup>	-
Anticapsular mAb	2 $\mu$ g	8047	0/5	<1	<1
Anti-rNspA	1:5	8047	1/6	0.2 <sup>b</sup>	2
Anti-rNspA	1:25	8047	1/5	0.4 <sup>b</sup>	4
Anti- <i>E.coli</i> control	1:5	8047	6/6	10 <sup>b</sup>	-

<sup>a</sup>  $p > 0.5$ , compared to geometric mean CFU/ml of control rats

<sup>b</sup>  $p < 0.001$ , compared to geometric mean CFU/ml of control rats

As can be seen, a dose of 2 $\mu$ g per rat of the positive anticapsular control was protective against both strains. A 1:5 or 1:25 dilution of anti-rNspA antiserum protected against bacteremia caused by strain 8047. Neither dilution was effective in preventing M986 bacteremia, however.

- Despite the positive conclusions of Martin *et al.*, therefore, NspA does not seem to be effective in preventing meningococcal B infection. Approximately one third of strains have decreased cell-surface expression of NspA epitopes when grown *in vitro*, are resistant to anti-NspA induced complement-mediated bacteriolysis, and are resistant to passive antiserum immunisation. These strains produce large amounts of capsular polysaccharide, and would thus be expected to have the greatest virulence. The ability of a vaccine containing only NspA to confer broad protective immunity against meningococcal B thus has to be doubted.

Compositions comprising NspA [SEQ IDs 4008-4033; Figure 29] therefore advantageously comprise further antigens. A preferred aspect of the invention is thus a combination of NspA protein with one or more further Neisserial antigens.

### Example 12 – NspA fragments

A model of the secondary structure of NspA is shown in Figure 32, containing eight transmembrane  $\beta$ -strands and 4 surface-exposed connecting loops. This fits the pattern of alternating hydrophobic and hydrophilic amino acids in NspA, which is characteristic of many  $\beta$ -barrel porins [Weiss *et al.* (1990) *FEBS Letts* 267:268-272]

The grey shaded areas in the model indicate segments that are >40% identical and >70% similar to encoded amino acid sequences of opacity proteins (Opa) from *N.meningitidis*, *N. gonorrhoeae*, *N. flavius*, *N. sicca*, and *H. influenzae* identified in a BLAST search of the non-redundant GenBank CDS. The alternating sequences are predicted amphiphilic  $\beta$ -strands; vertical segments correspond to transmembrane segments; the top of the figure corresponds to surface exposed segments, labelled as loops 1 to 4 .

According to Martin *et al.*, the only significant homology between the deduced amino acid sequence of NspA and those of other proteins are weak homologies with the Neisseria opacity protein (Opa) family in two small segments (~20 amino acids) near the C-terminal end of the protein . However, separate comparisons of the N- and C-termini of NspA with GenBank reveals a high degree of homology (>40 % identity and >70% similarity) between NspA and Opa proteins from *N.meningitidis*, *N. gonorrhoeae*, *N. flavius*, *N. sicca*, and *H. influenzae*. The Opa proteins are thought to be integral membrane proteins that have eight transmembrane segments and a  $\beta$ -barrel topology in the membrane similar to that of porin [Merker *et al.* (1997) *Mol. Microbiol.* 23:281-293]. The presence of NspA in detergent-insoluble membrane preparations indicate that NspA is located in the outer membrane, which would be consistent with the Opa-like membrane topology shown in the model. In addition, the segments of NspA that are most homologous to those of the Opa proteins are the putative transmembrane segments indicated in the shaded areas of Figure 32.

The opacity proteins of Neisseria can, under certain circumstances, elicit protective antibody. However, problems with limited antibody accessibility of the opacity proteins in encapsulated bacteria, variability of amino acid sequences in exposed loop segments, and phase variation of protein expression during clinical infection, have limited the ability of Opa to elicit protective antibody consistently [Malorny *et al.* (1998) *J. Infect. Dis.* 172:1279-89]. In contrast, there appears to be little or no sequence variation in the surface exposed loops of NspA in Figure 32. However, it was recently reported that a panel of anti-*N.meningitidis* NspA monoclonal antibodies that reacted with all meningococcal strains tested reacted with only a limited number

of *N.gonorrhoeae* strains, even though the respective amino acid sequences in the two species are 92% identical. When the respective NspA sequences of the meningococcal and gonococcal strains are compared (Figure 29), all of the respective amino acid differences result in changes in hydrophobicity or charge, and are located in the putative surface exposed connecting loops (Figure 32). This finding suggests that the connecting loops in NspA, which are highly conserved in *N.meningitidis*, may be important epitopes for antibodies that bind to native NspA. These segments of the molecule, therefore, would appear to be of greatest interest with respect to interacting with protective antibody. However, the putative surface loops of NspA are relatively small (10-14 amino acids) compared to, for example, the highly immunogenic external loops of PorA and Opc (24 to 45 amino acids). The shorter length of the loops may limit the accessibility of NspA surface epitopes for binding interactions with serum antibody, especially in the presence of abundant capsular polysaccharide.

Accordingly, the invention provides the fragments of NspA that are exposed on the cell-surface in Figure 32, namely SSSLGSAKG, NYKAPSTDFKLY, NRASVDLGGSDSFSQT, and NYIGKVNTVKNVRSG, and also provides corresponding fragments from allelic variants of NspA. In addition, the invention provides sub-sequences of these fragments, comprising 7 or more contiguous amino acids from the fragments. The invention further provides proteins comprising these fragments. Nucleic acid encoding these fragments and proteins is also provided.

These NspA fragments, proteins comprising the fragments, and nucleic acid, may be used in the compositions of the invention, in particular as substitutes for full-length NspA. In a further aspect, these fragments, proteins and nucleic acids may be used as products in isolation, that is to say they need not exclusively be used in combination with other biological molecules.

It will be appreciated that the invention has been described by means of example only, and that modifications may be made whilst remaining within the spirit and scope of the invention.

**Reactivity of anti-rNspA polyclonal antisera with native NspA exposed on the surface of live, encapsulated, *Neisseria meningitidis* B bacteria in relation to susceptibility to bacteriolysis and capsular production.**

Meningococcal B Strains						
Strain	Country	Year	Serologic Classification	ET Complex	NspA Cell	Polysaccharide
					Surface Reactivity <sup>c</sup>	Activity <sup>d</sup> (1/titer)
SWZ107 <sup>a</sup>	Switzerland	1980	4:P1.2	104	Positive	28±4
NG6/88 <sup>a</sup>	Norway	1988	NT: P1.1	173	Positive	115±23
CU385 <sup>b</sup>	Cuba	1980	4: P1.15	5	Positive	116±1
IH5341	Finland	1985	15:P1.7,16	ND	Positive	176±61
BZ198	Netherlands	1986	NT: P-	154	Positive	362±1
NMB	US	1968	2b:P1.2,5	ND	Positive	244±20
8047	US	1978	2b: P1.2	ND	Positive	1125±50
H44/76	Norway	1976	15: P1.7, 16	5	Positive	99±16
1000 <sup>a</sup>	USSR	1989	NT:P1.5	61	Positive	287±12
S3446 <sup>b</sup>	US	1972	14: P1.23, 14	11 (A1 cluster)	Positive	585±151
H355 <sup>b</sup>	Norway	1973	15: P1.15	11 cluster	Positive	656±141
BZ232 <sup>a</sup>	Netherlands	1964	NT:P1.2	76	Negative	1493±18
NG3/88 <sup>a</sup>	Norway	1988	8: P1.1	A4 cluster	Negative	498±105
MC58	UK	1985	15:P1.7,16b	5	Negative	627±121
M136 <sup>b</sup>	US	1968	11: P1.15	D1 cluster	Negative	1056±81
M986 <sup>b</sup>	US	1963	2a: P1.5, 2	B2 cluster	Negative	1442±206
NGP165	Norway	1974	NT:P1.2	37	Negative	138±6

<sup>a</sup> Denotes strains that have been characterized further by multilocus sequence typing [Maiden, 1998].

<sup>b</sup> Denotes strains obtained from the Frasch collection, US FDA. 8047 was obtained from W. Zollinger, Walter Reed Army Institute of Research, Washington, D.C. MC58 is the strain selected by TIGR for genomic sequencing. J351 was obtained from M. Sarvas, National Public Health Institute, Helsinki, Finland. The remaining strains are from the collection described by Seiler *et al.* [Seiler, 1996]. ET data are from Caugnant *et al.* [J. Infect. Dis. (1990) 162:867-874], and Seiler *et al.*

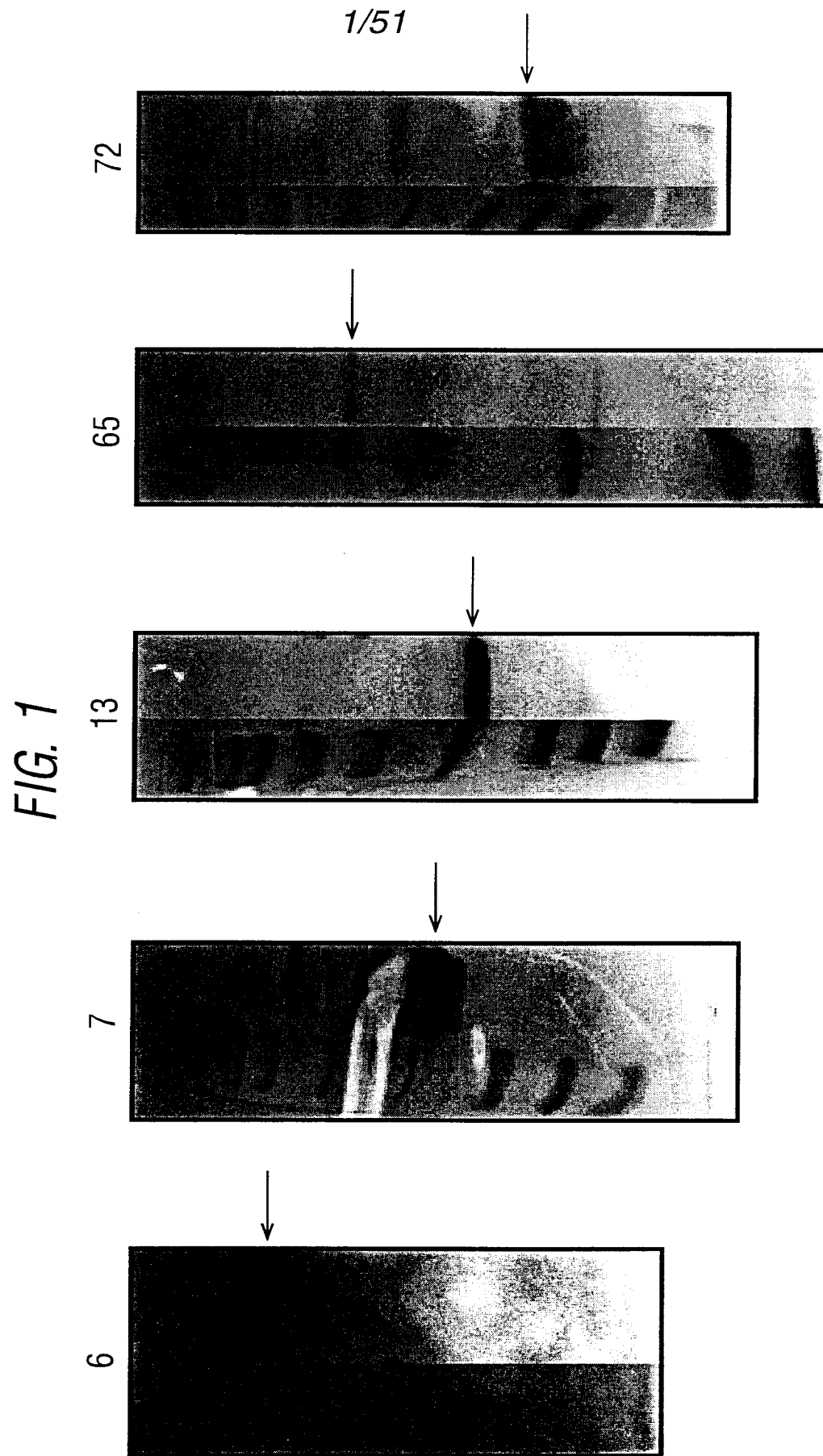
<sup>c</sup> By indirect fluorescence flow cytometry with anti-rNspA antisera.

<sup>d</sup> Dilution of anti-rNspA antisera that when incubated for 60 min. with bacterial cells and 20% human complement yielded ≥50% decrease in CFU/ml, compared to that at time 0. "Static" refers to strains that were inhibited but not killed in the assay (≥50% but <100 % survival at 60 mins).

<sup>e</sup> Titre defined as dilution of capsule extract giving 50% inhibition of antibody binding to meningococcal B polysaccharide antigen in an ELISA.

**CLAIMS**

1. A composition comprising a first biological molecule from a *Neisseria* bacterium and a second biological molecule from a *Neisseria* bacterium.
2. The composition of claim 1, wherein the first biological molecule is selected from the group consisting of SEQ IDs 1-8376.
3. The composition of claim 2, wherein the first biological molecule is selected from the group consisting of SEQ IDs 1-4002.
4. The composition of any preceding claim, wherein the second biological molecule is selected from the group consisting of SEQ IDs 1-8376.
5. The composition of claim 4, wherein the second biological molecule is selected from the group consisting of SEQ IDs 1-4002.
6. The composition of any one of claims 1 to 4, comprising two or more biological molecules selected from SEQ IDs 1-4002.
7. The composition of any one of claims 1 to 4, comprising one or more biological molecules selected from SEQ IDs 1-4002 combined with one or more biological molecules selected from SEQ IDs 4003-8376.
8. The composition of any one of claims 1 to 4, comprising one or more biological molecules selected from SEQ IDs 1-4002 & 4057-8376 combined with the NspA protein disclosed in WO96/29412, preferably in mature form.
9. The composition of any one of claims 1 to 4, comprising one or more biological molecules selected from SEQ IDs 1-8376 combined with 953 protein.
10. The composition of any preceding claim for use as a medicament



2/51



147



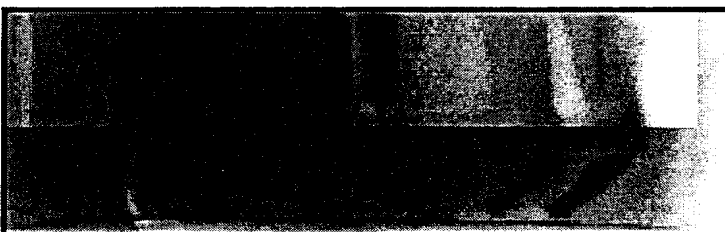
143



137



105



73

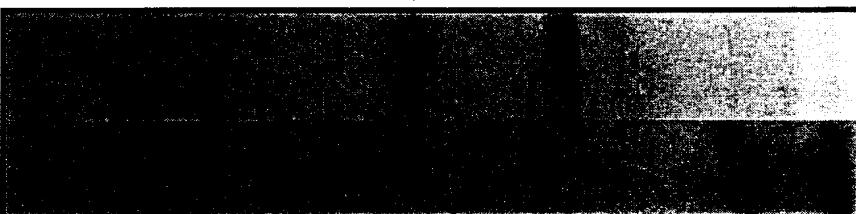


FIG. 1 (CONTD.)



3/51

FIG. 2A

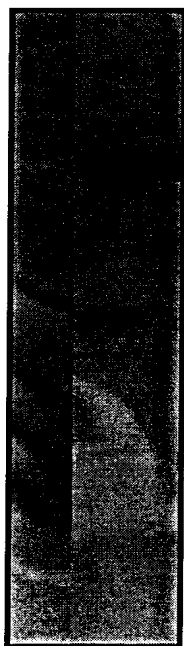
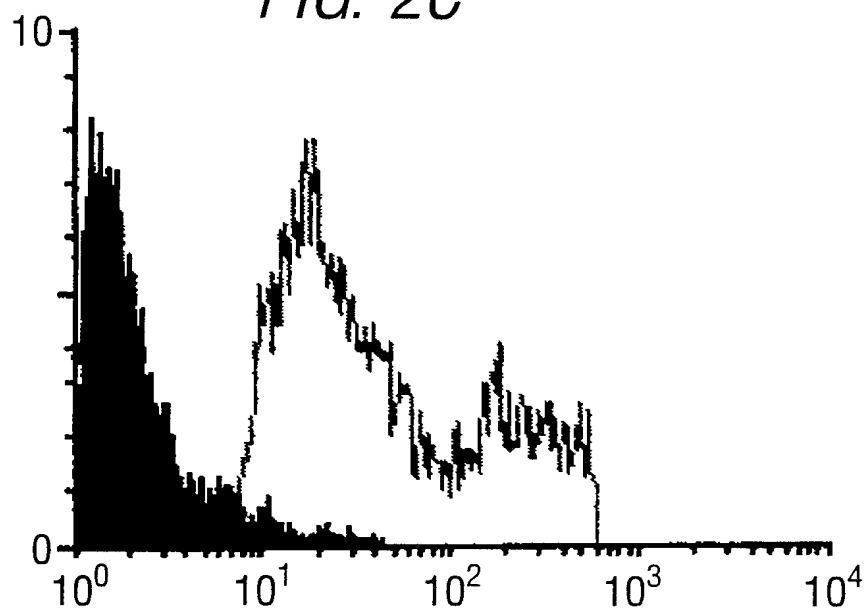


FIG. 2B



FIG. 2C



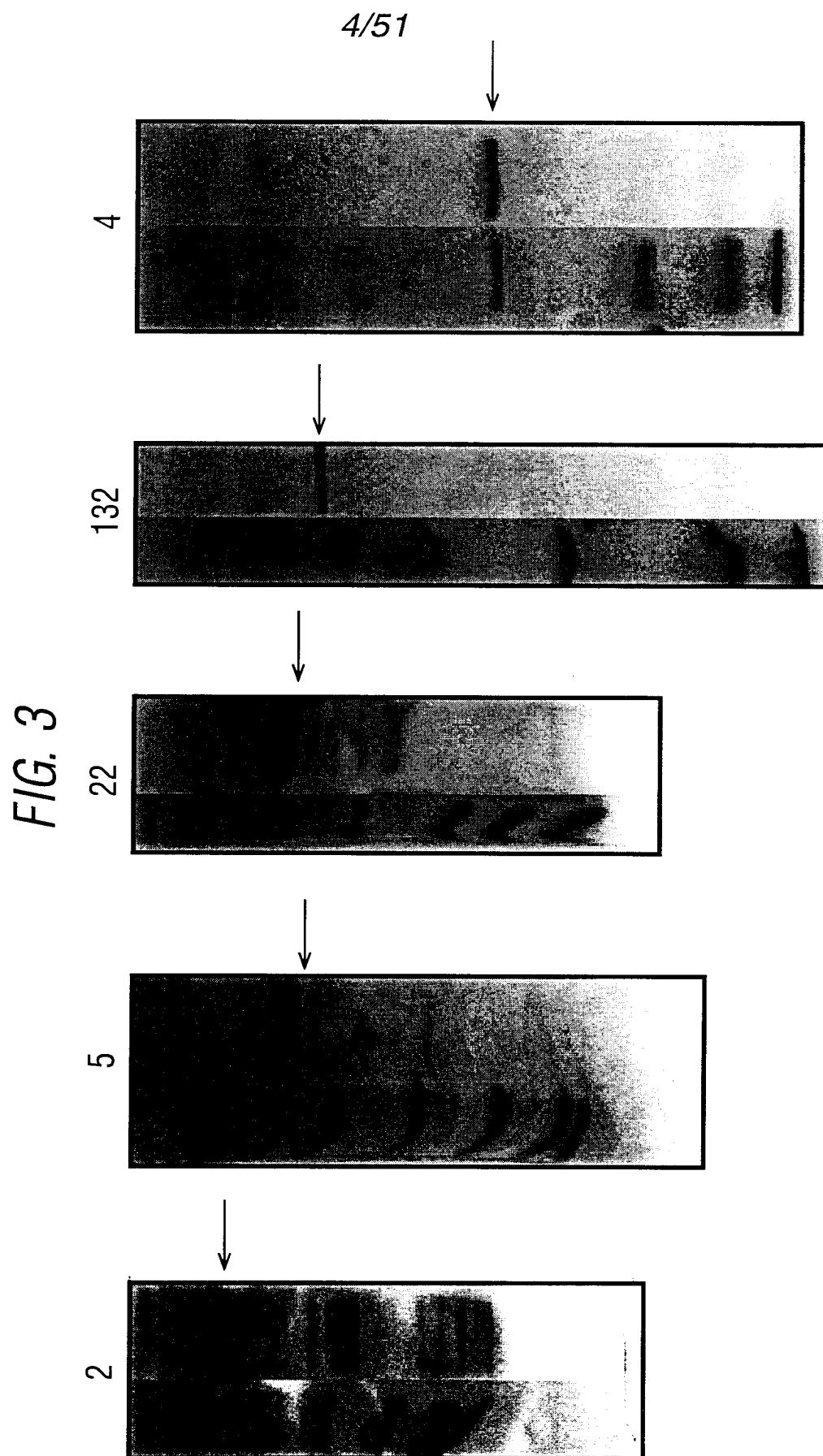


FIG. 4  
ORF2

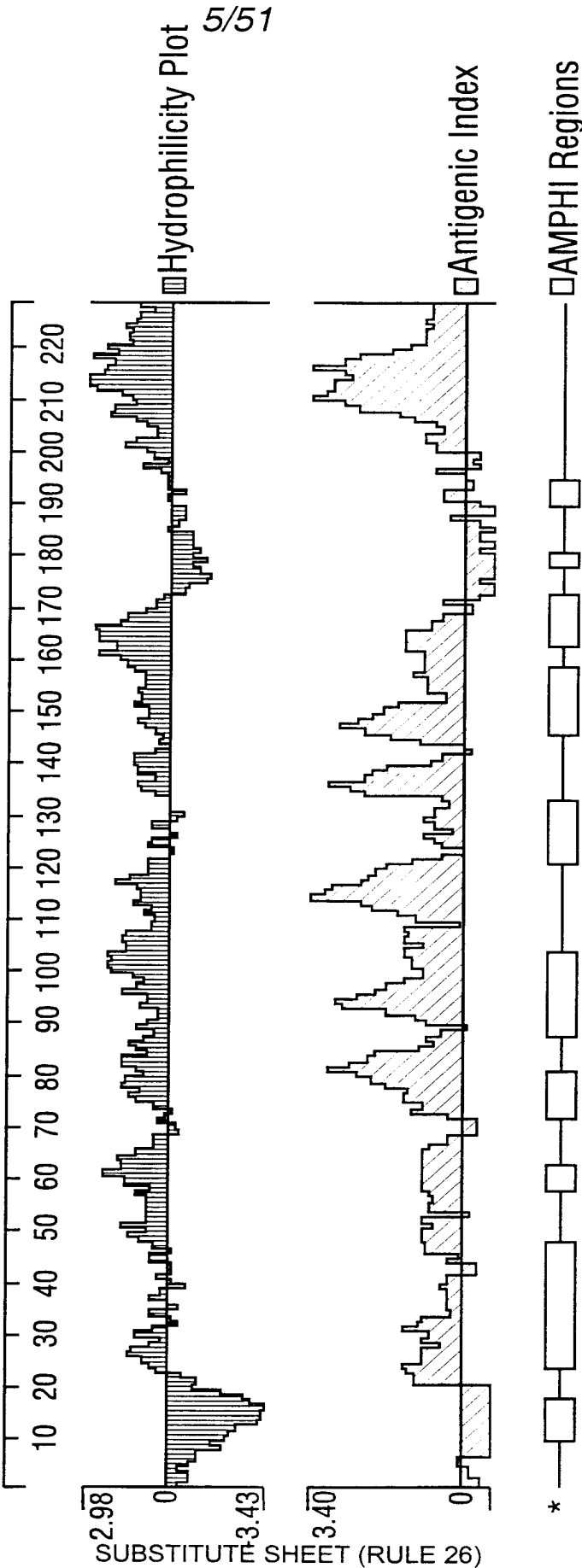
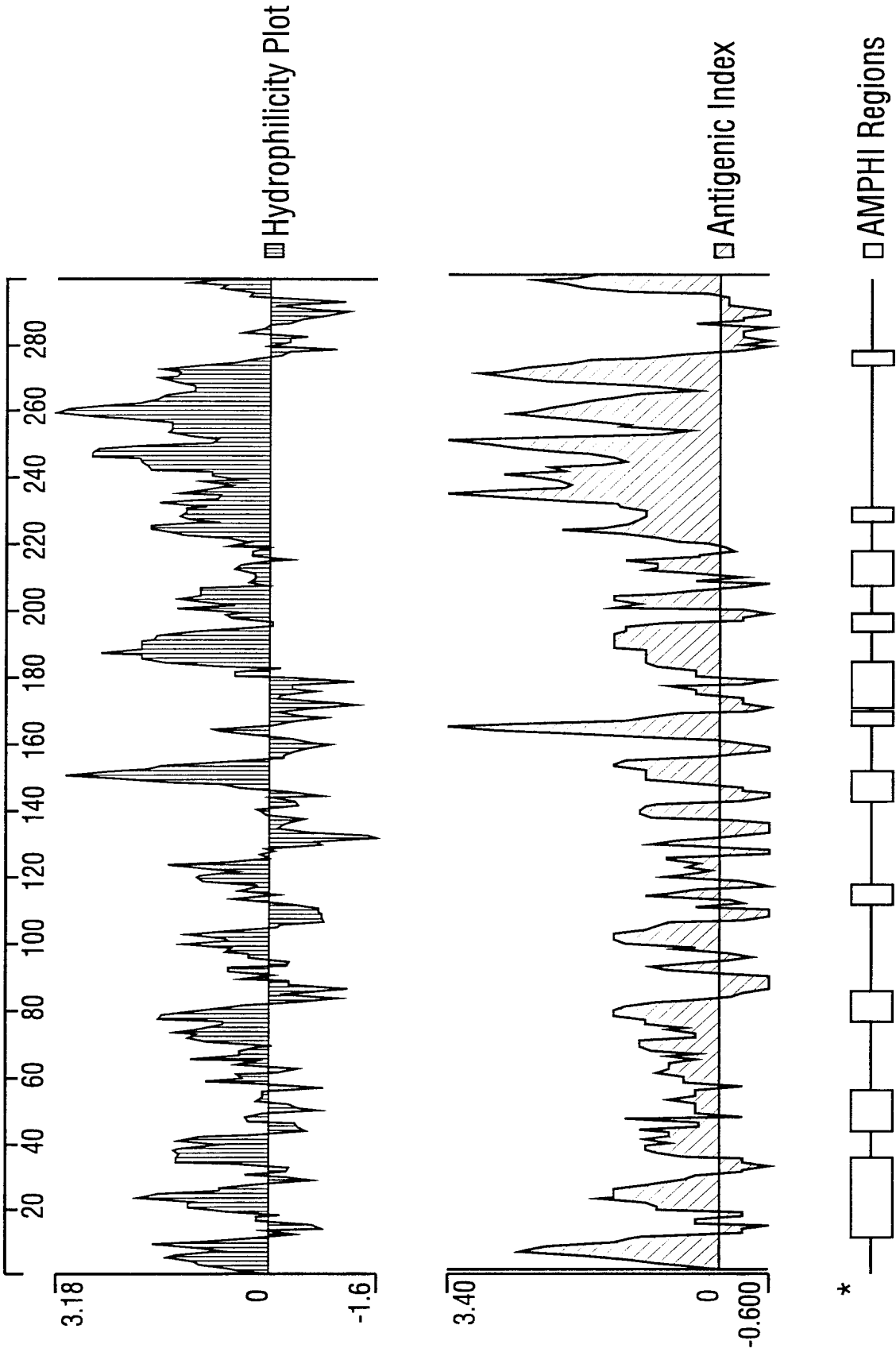


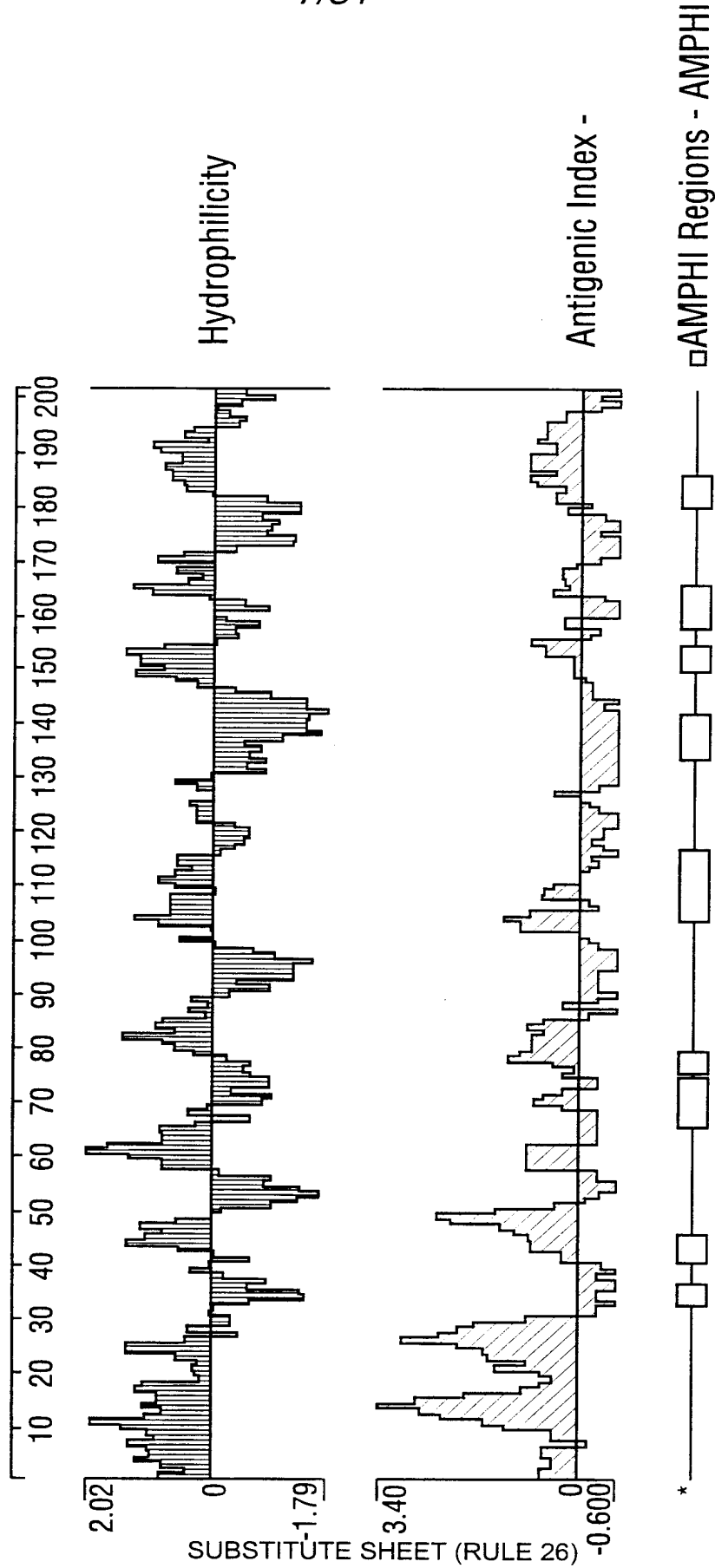
FIG. 5

ORF5

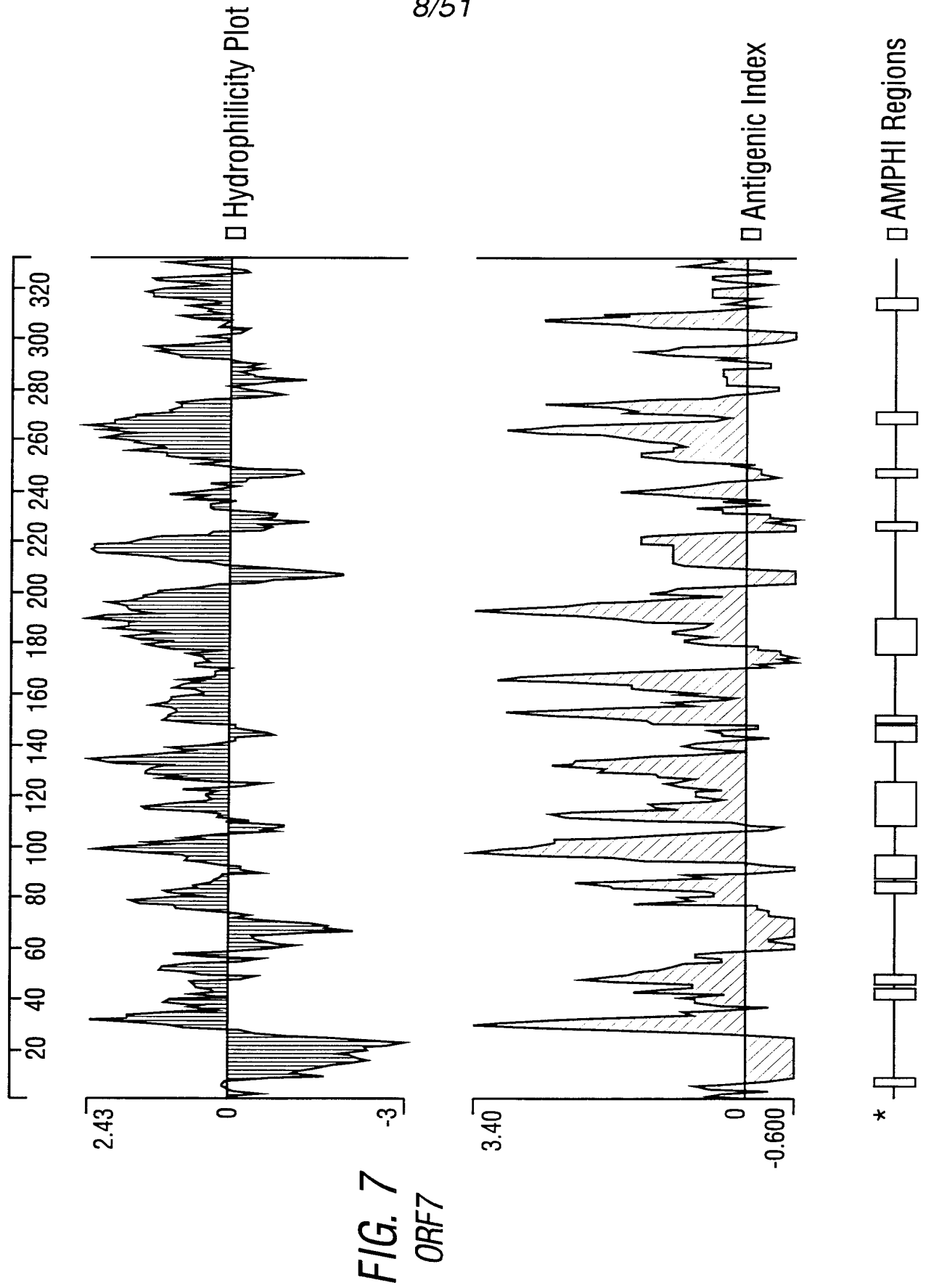


7/51

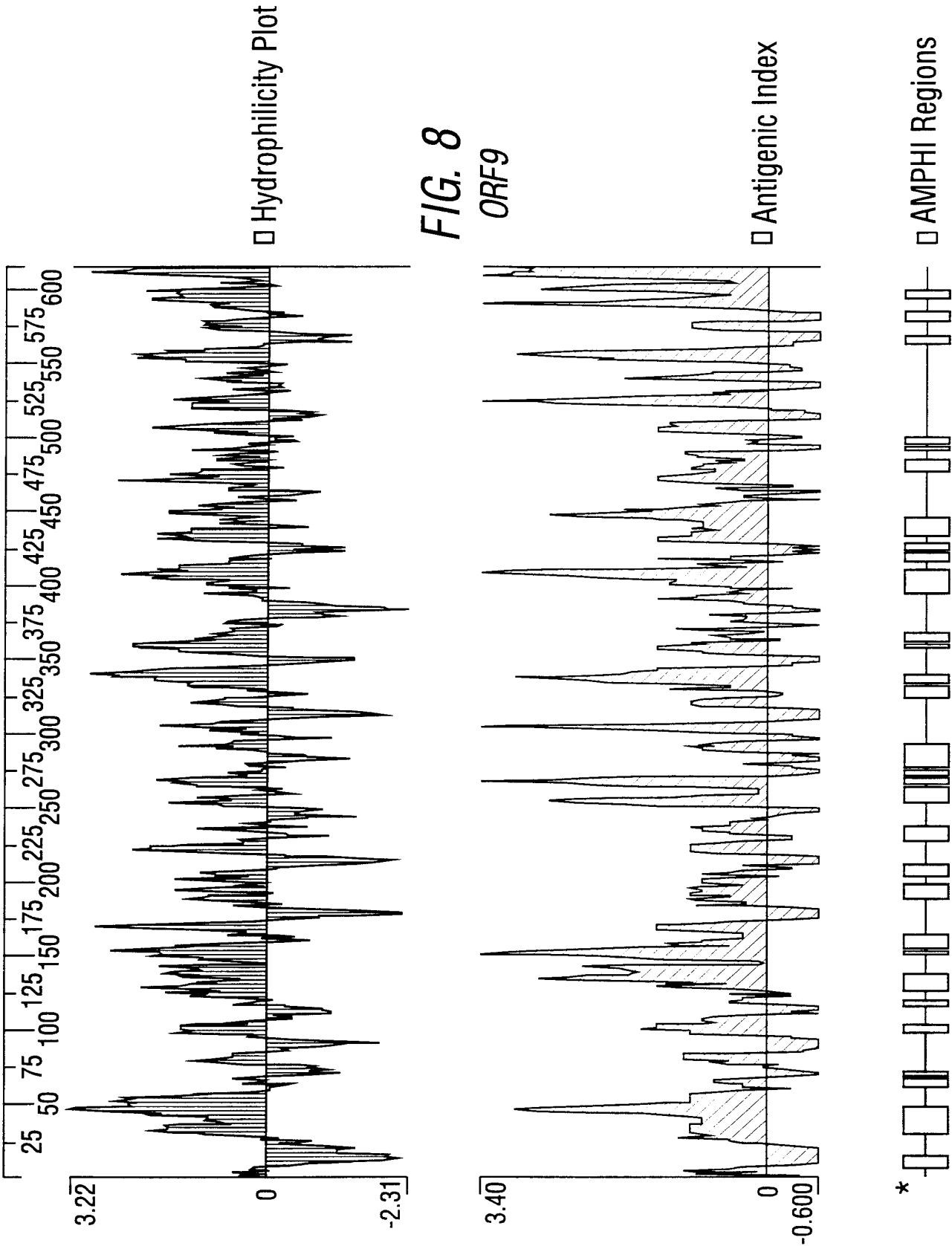
FIG. 6  
ORF6a



8/51

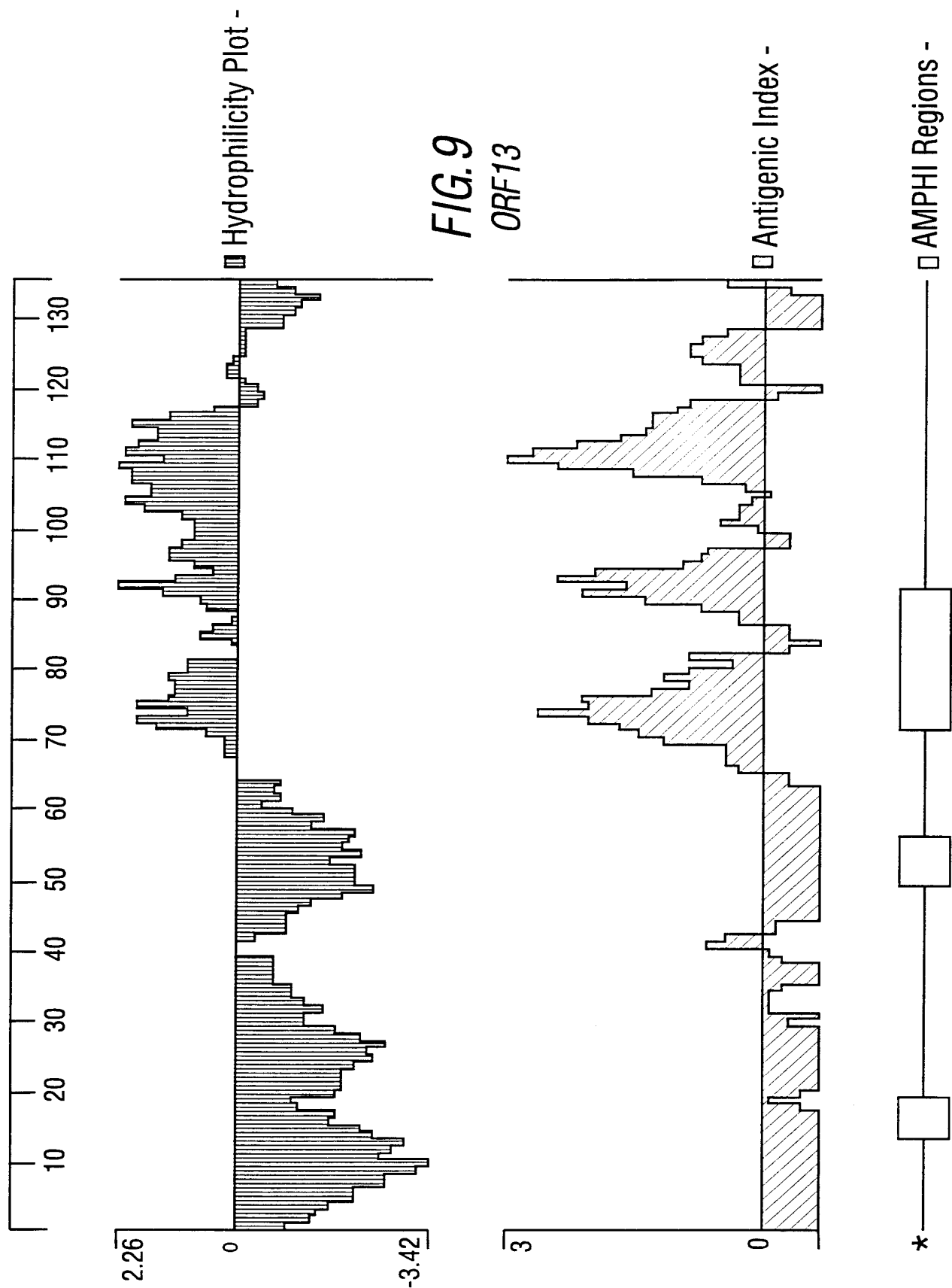


9/51



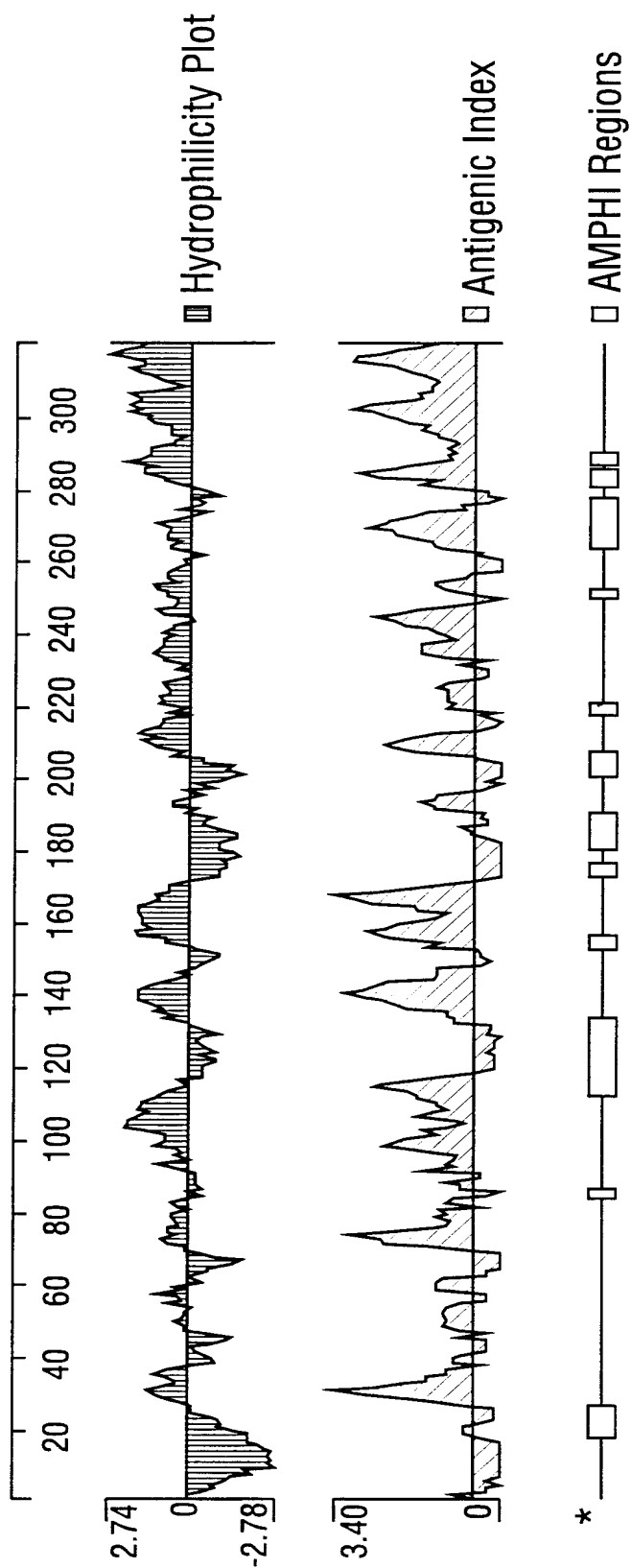


10/51



11/51

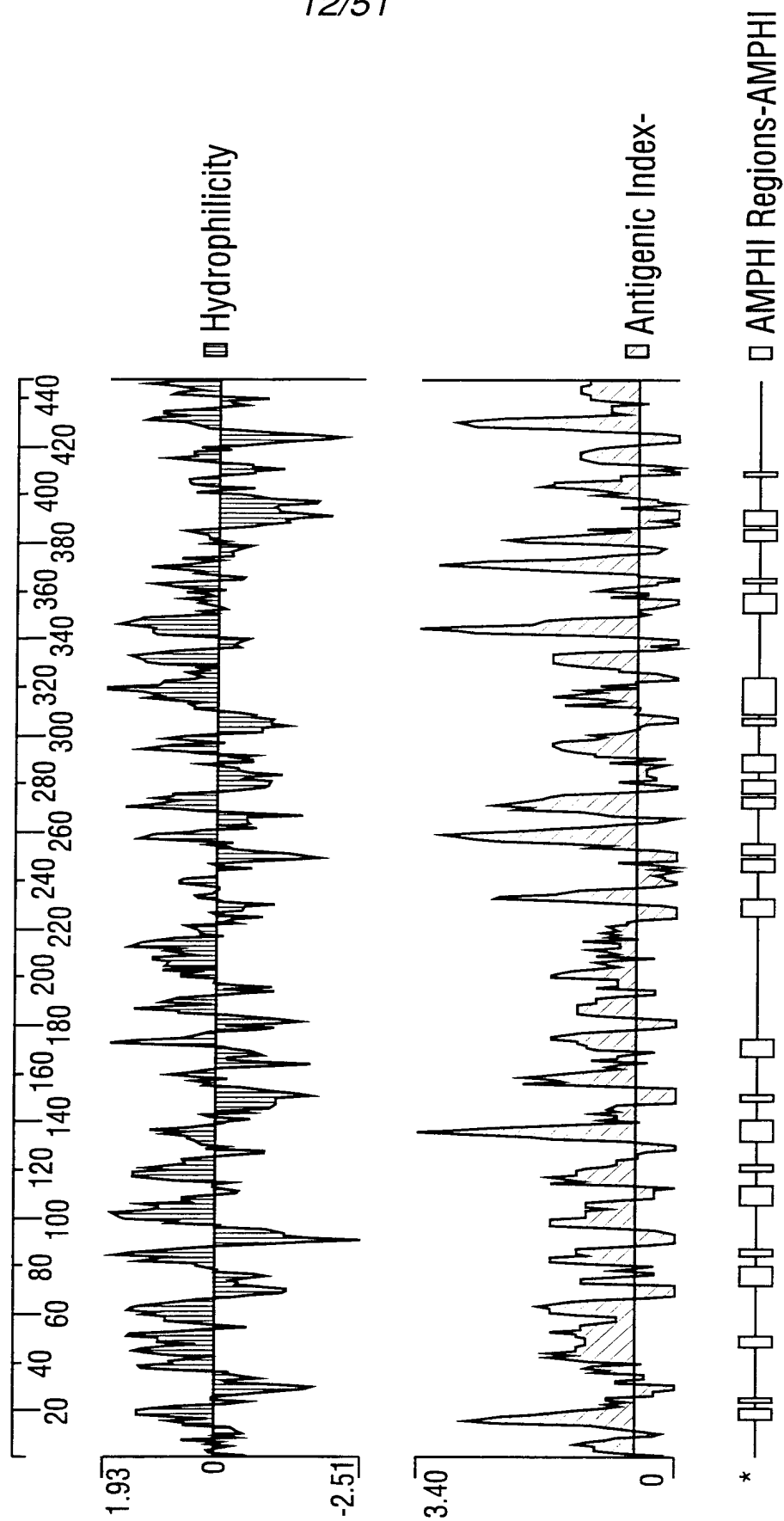
**FIG. 10**  
**ORF15**

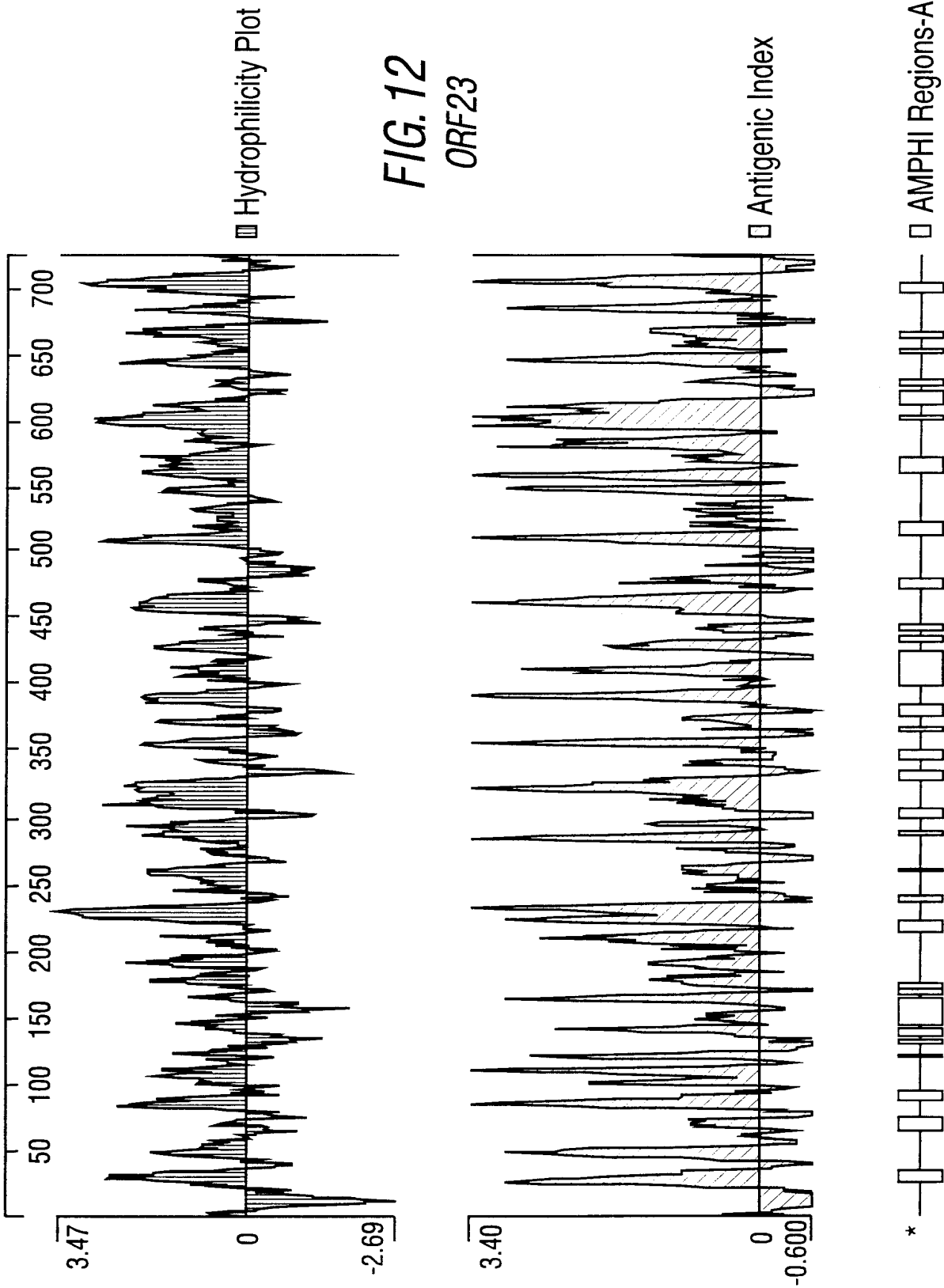


12/51

FIG. 11

ORF22





14/51

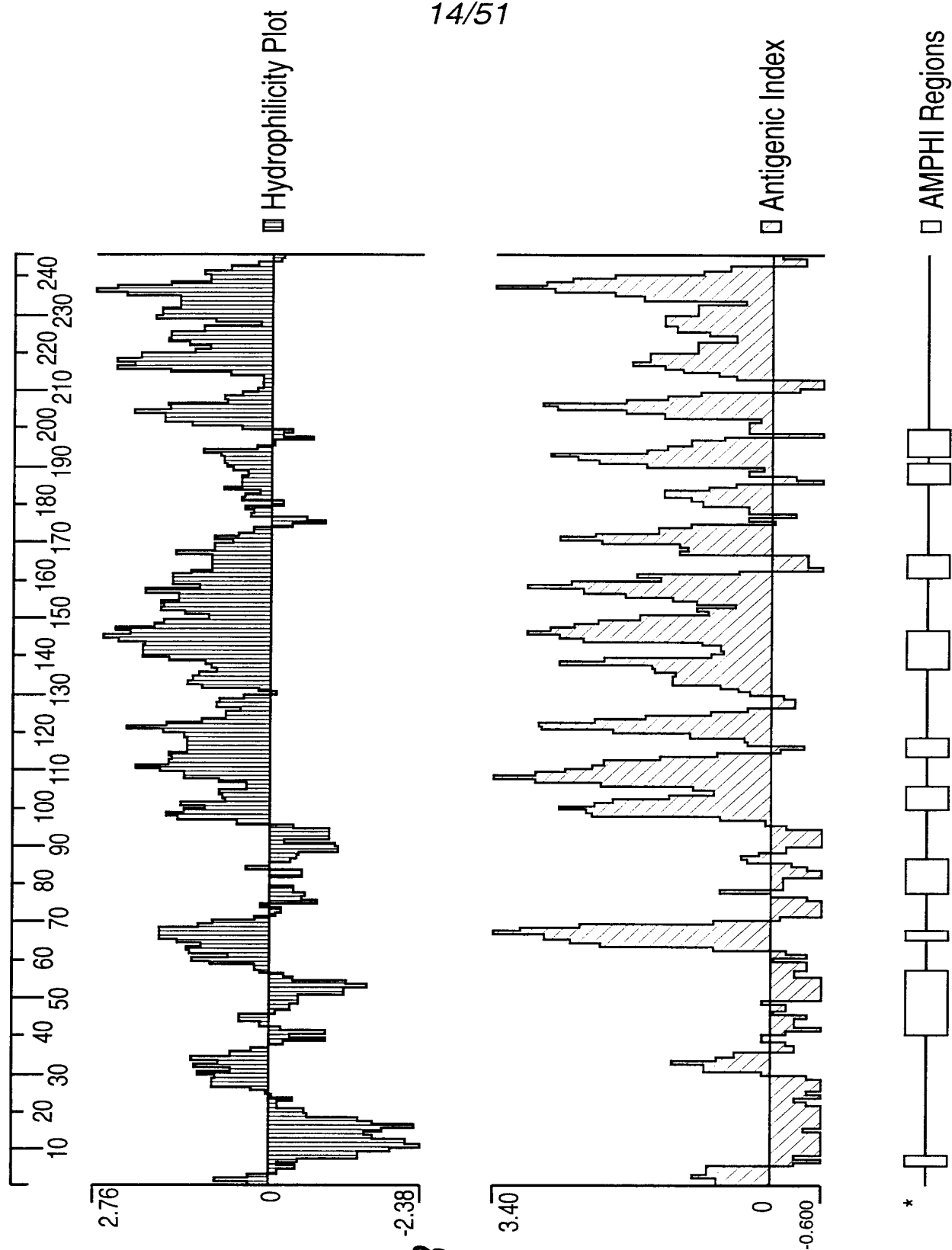


FIG. 14  
ORF28

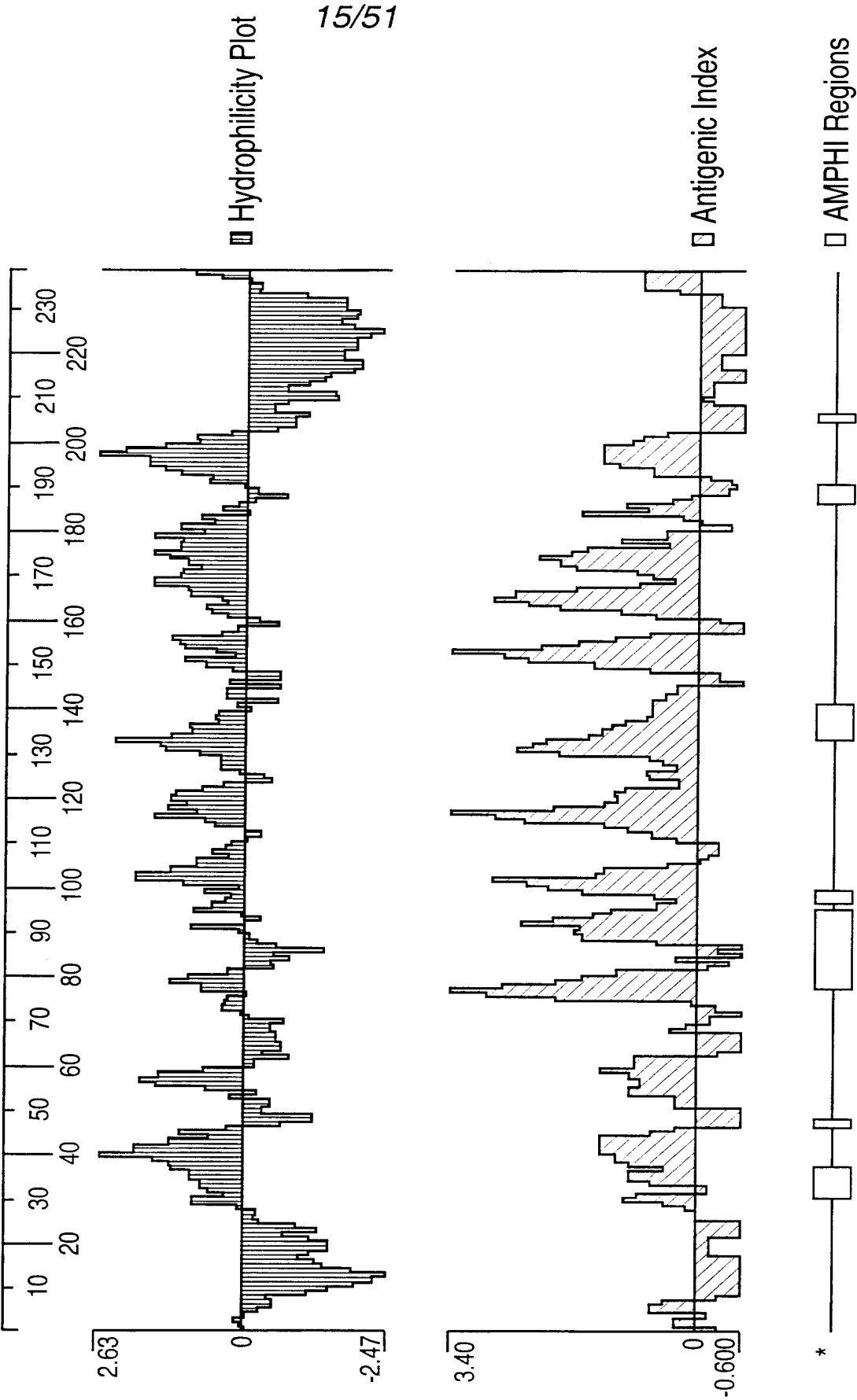


FIG. 15  
ORF32

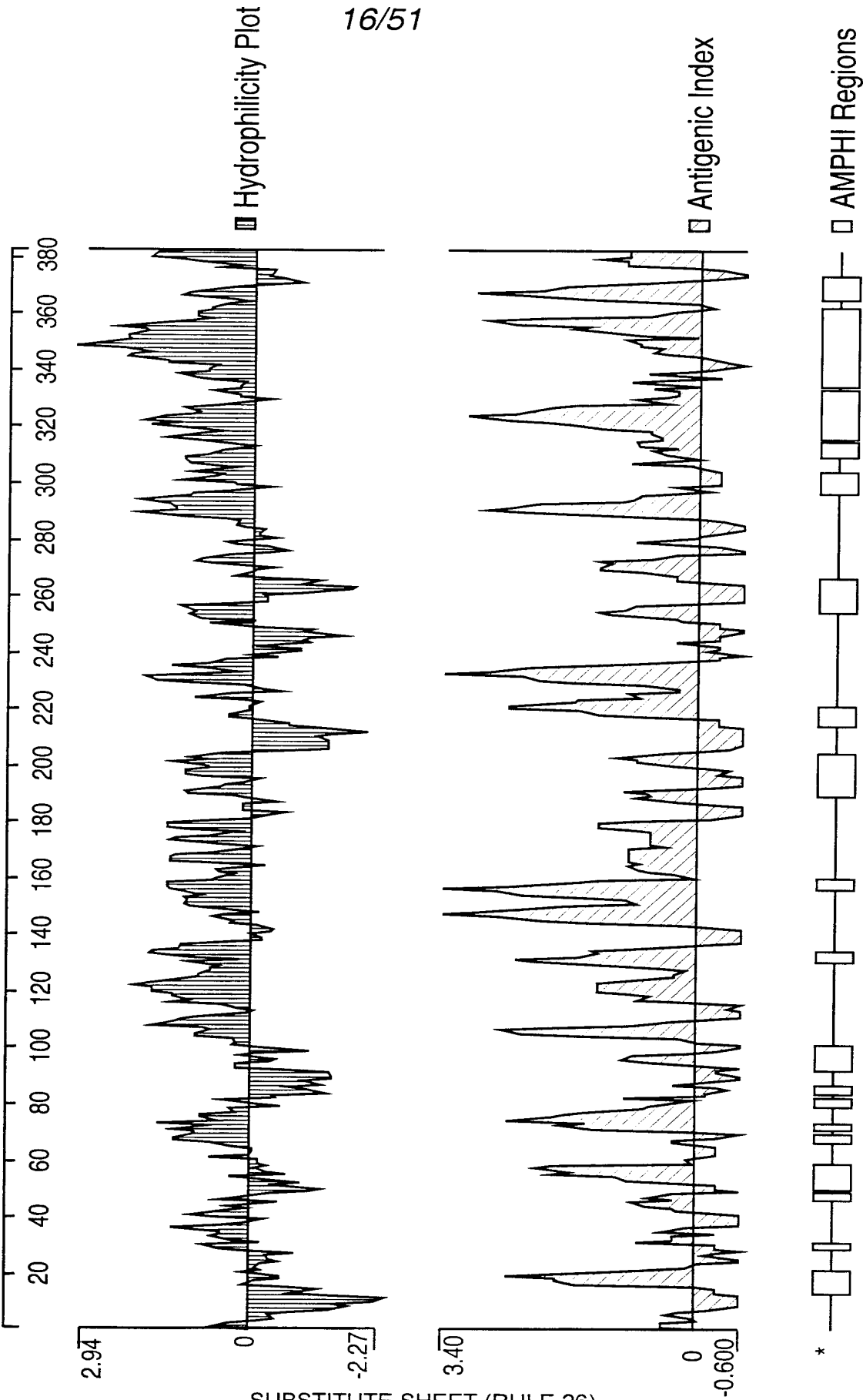
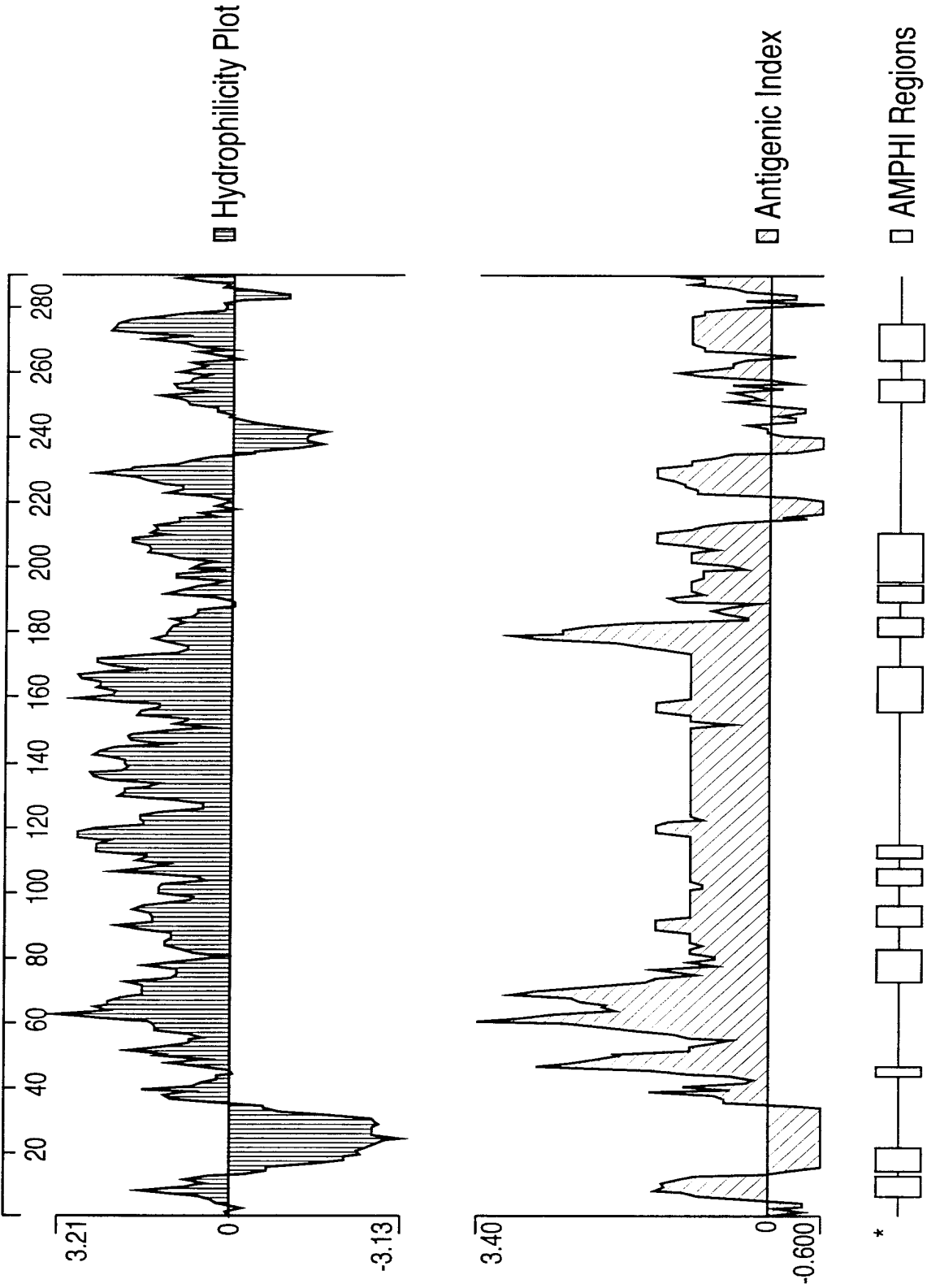


FIG. 16  
ORF65





18/51

FIG. 17

ORF72

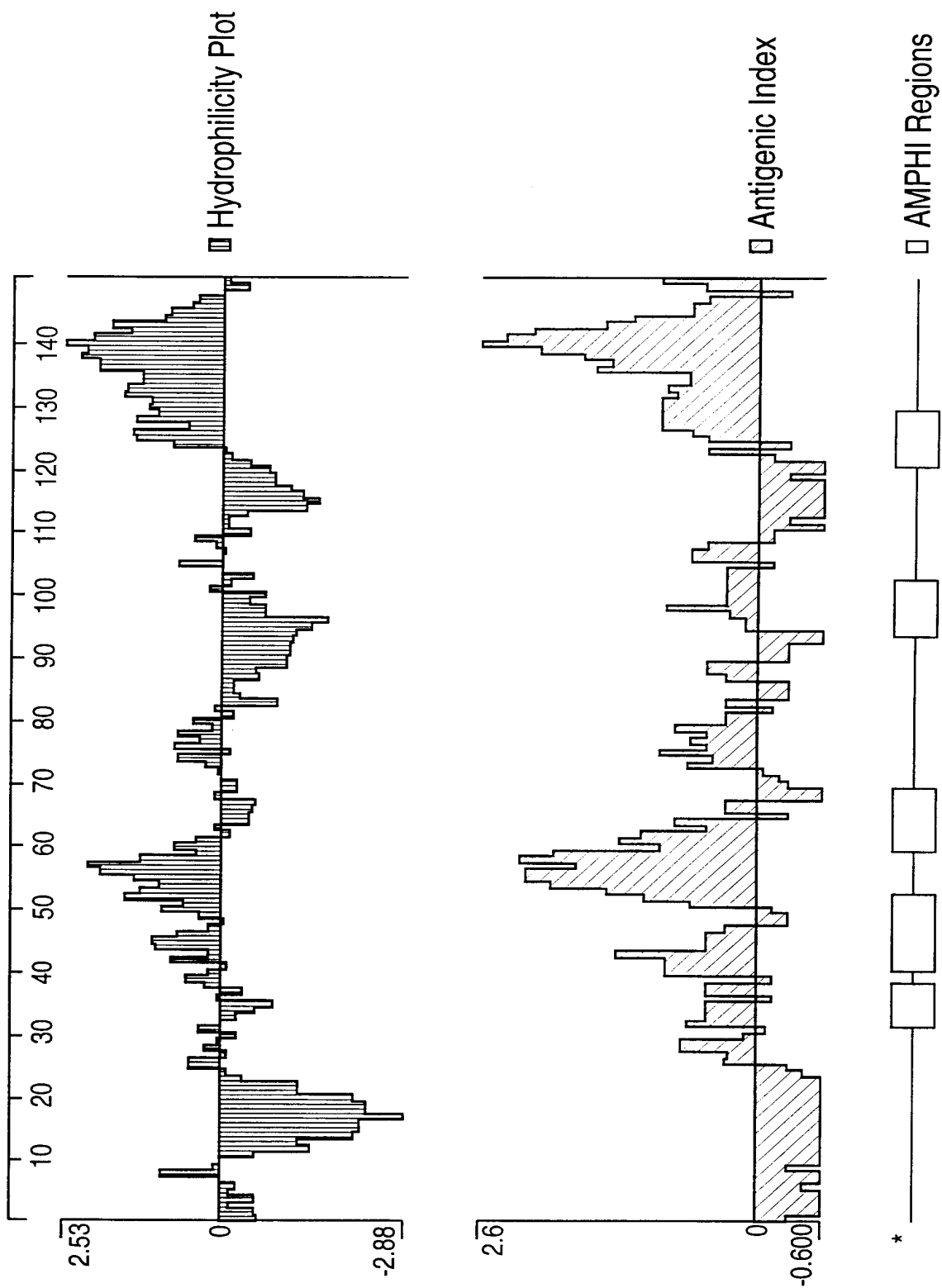


FIG. 18  
ORF73

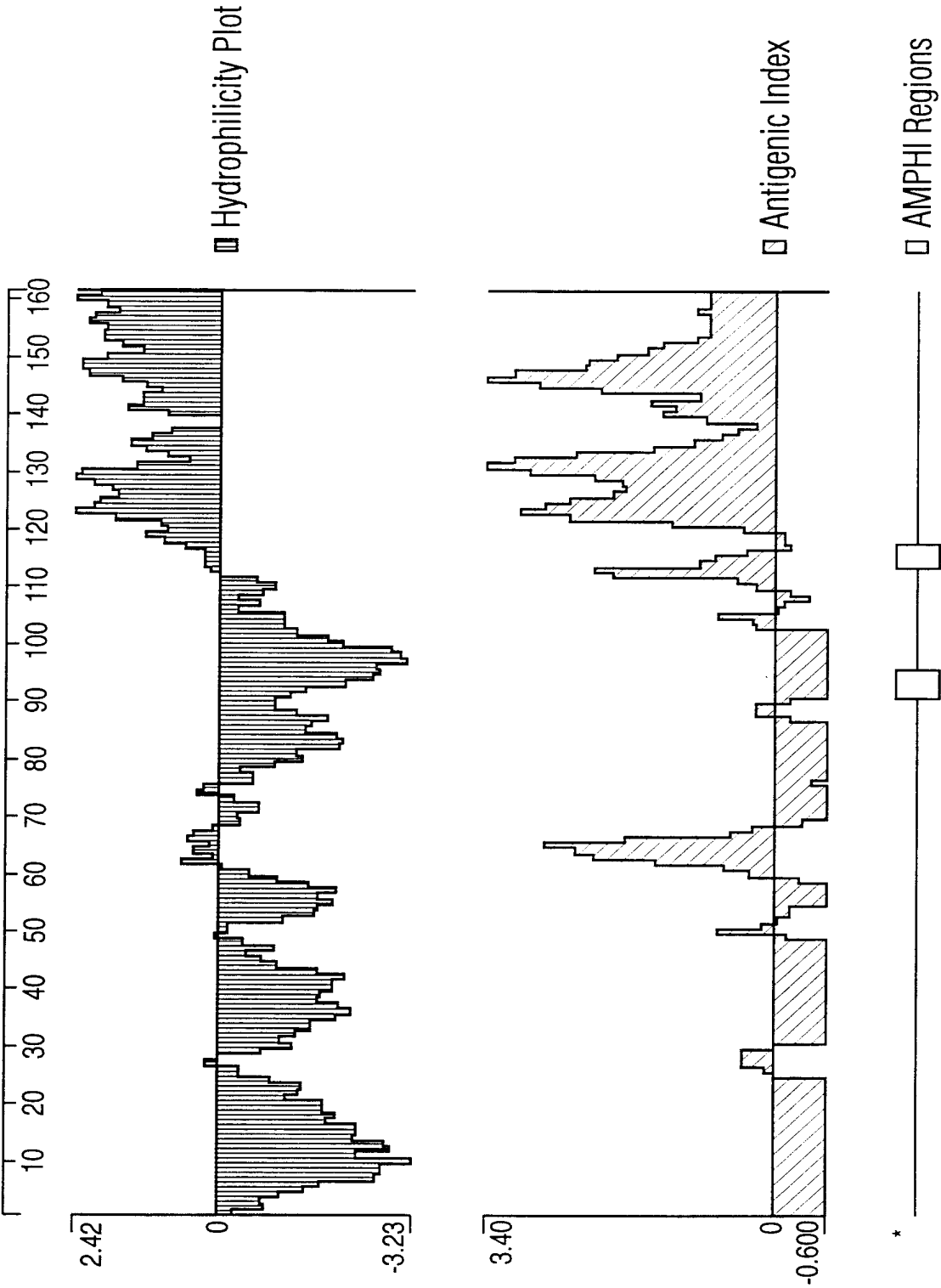
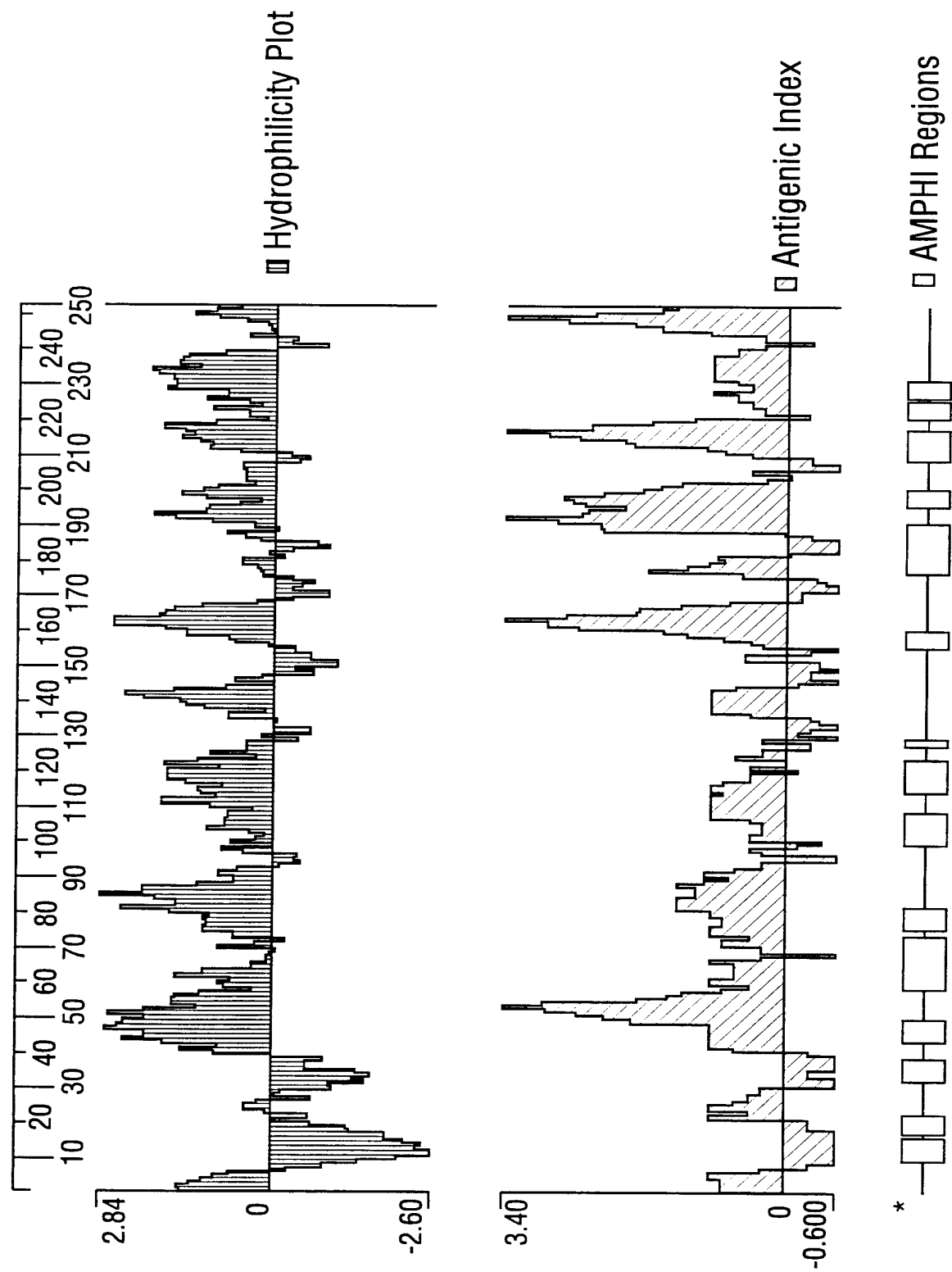


FIG. 19  
ORF76



21/51

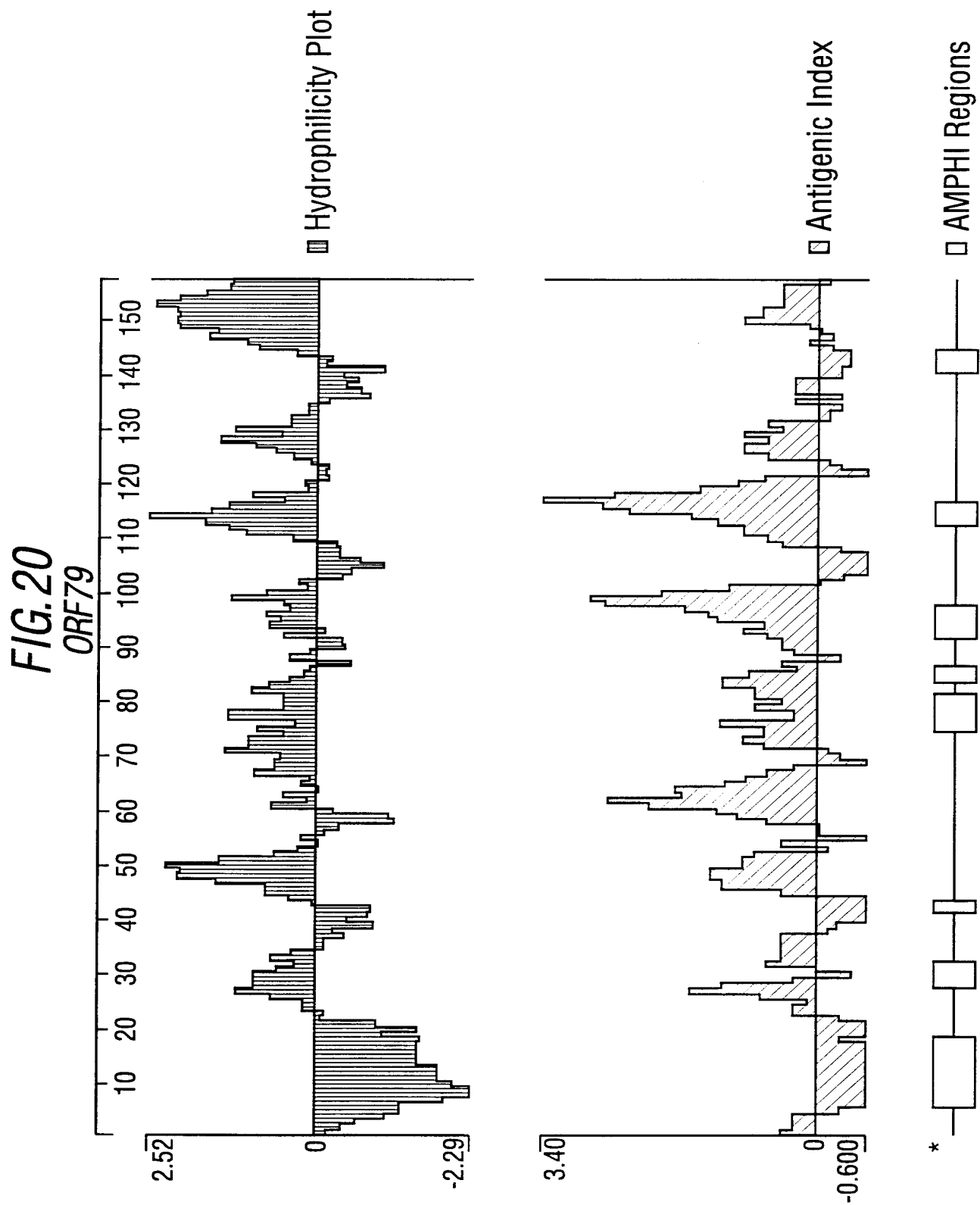


FIG. 21  
ORF89

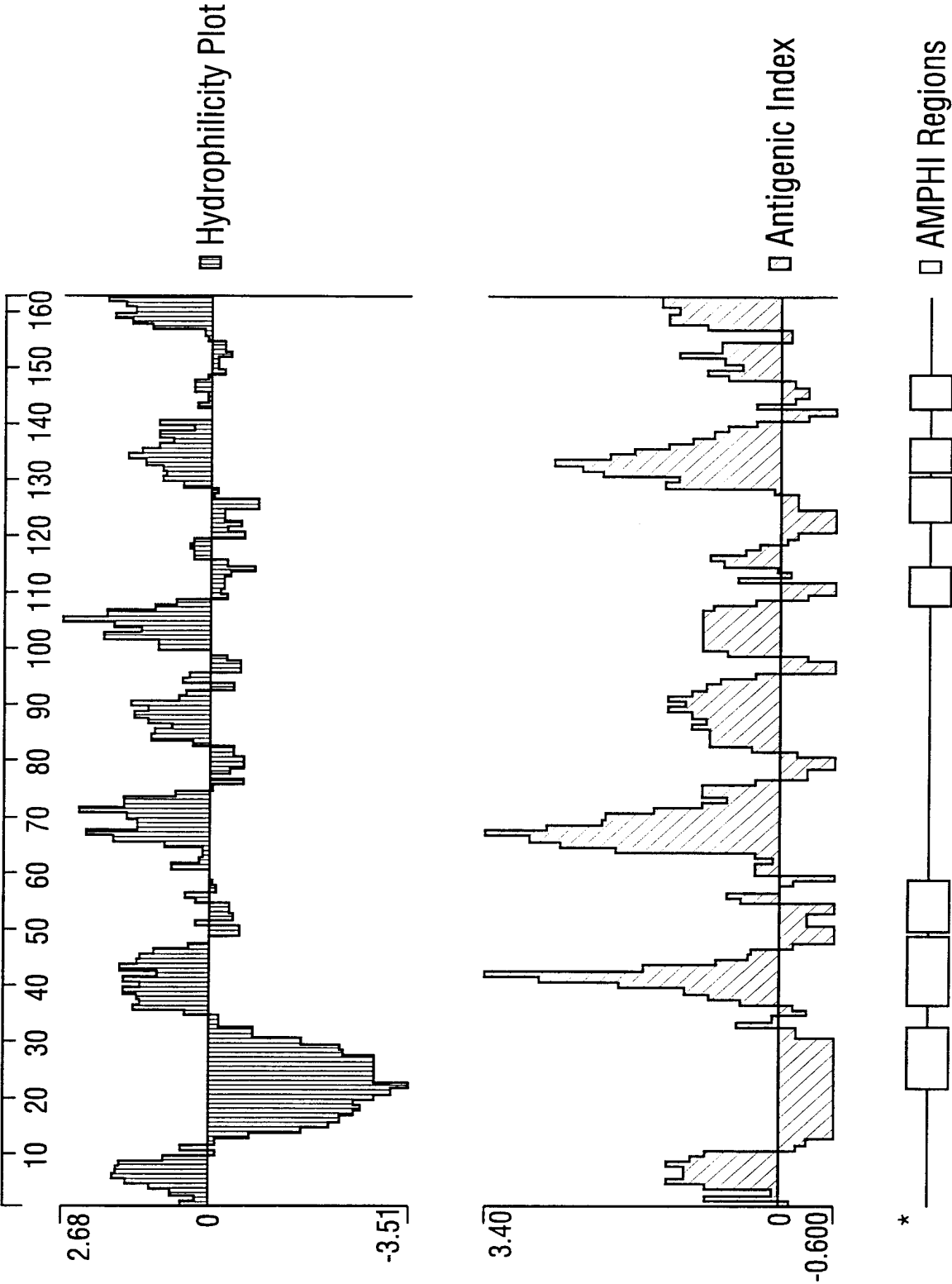


FIG. 22  
ORF105

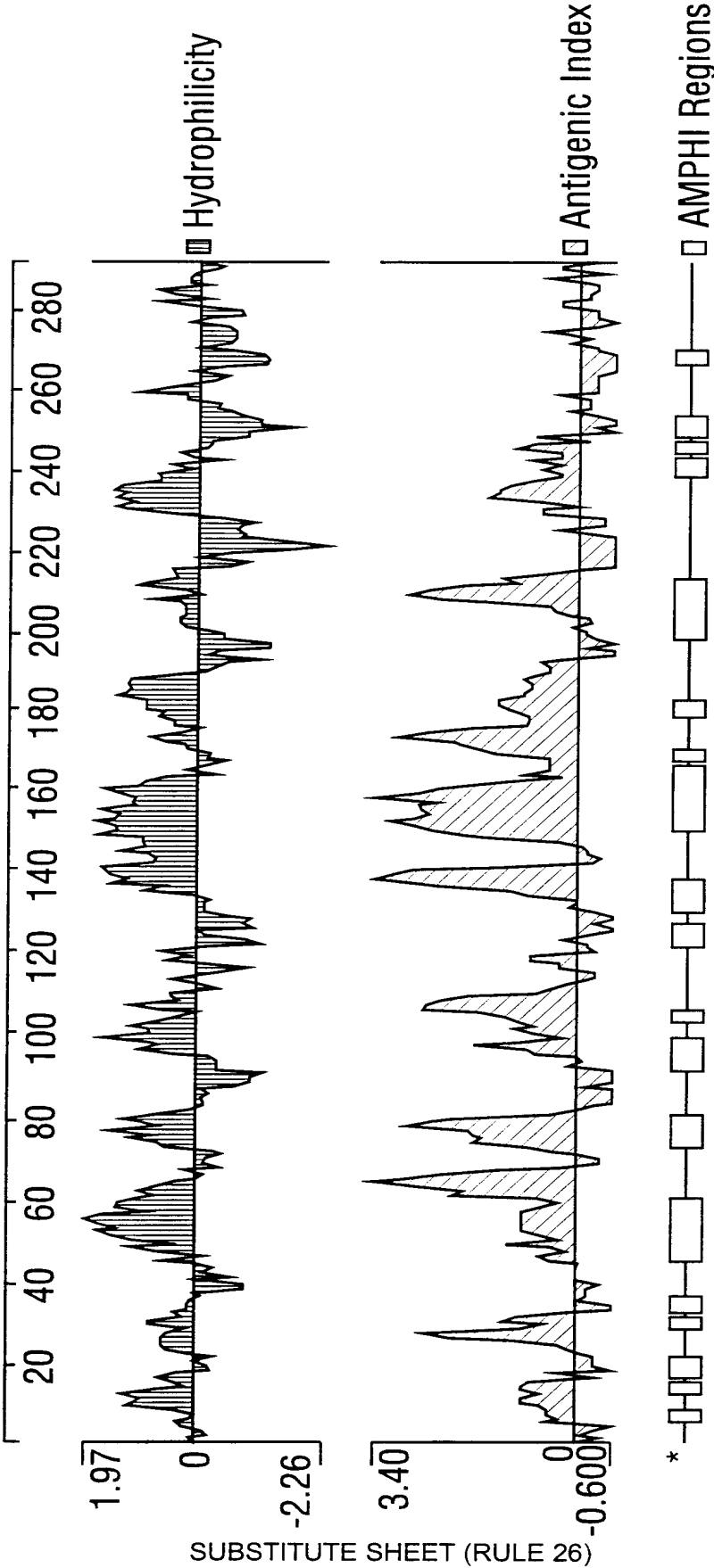


FIG. 23  
ORF106

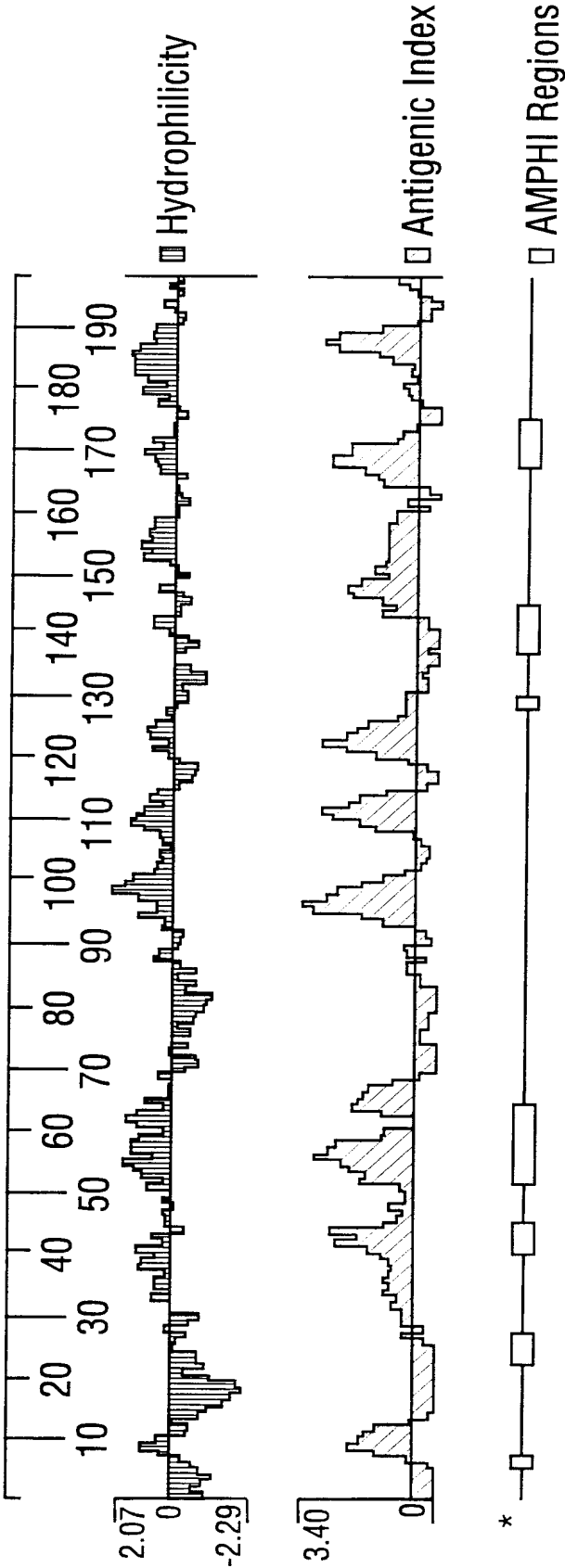
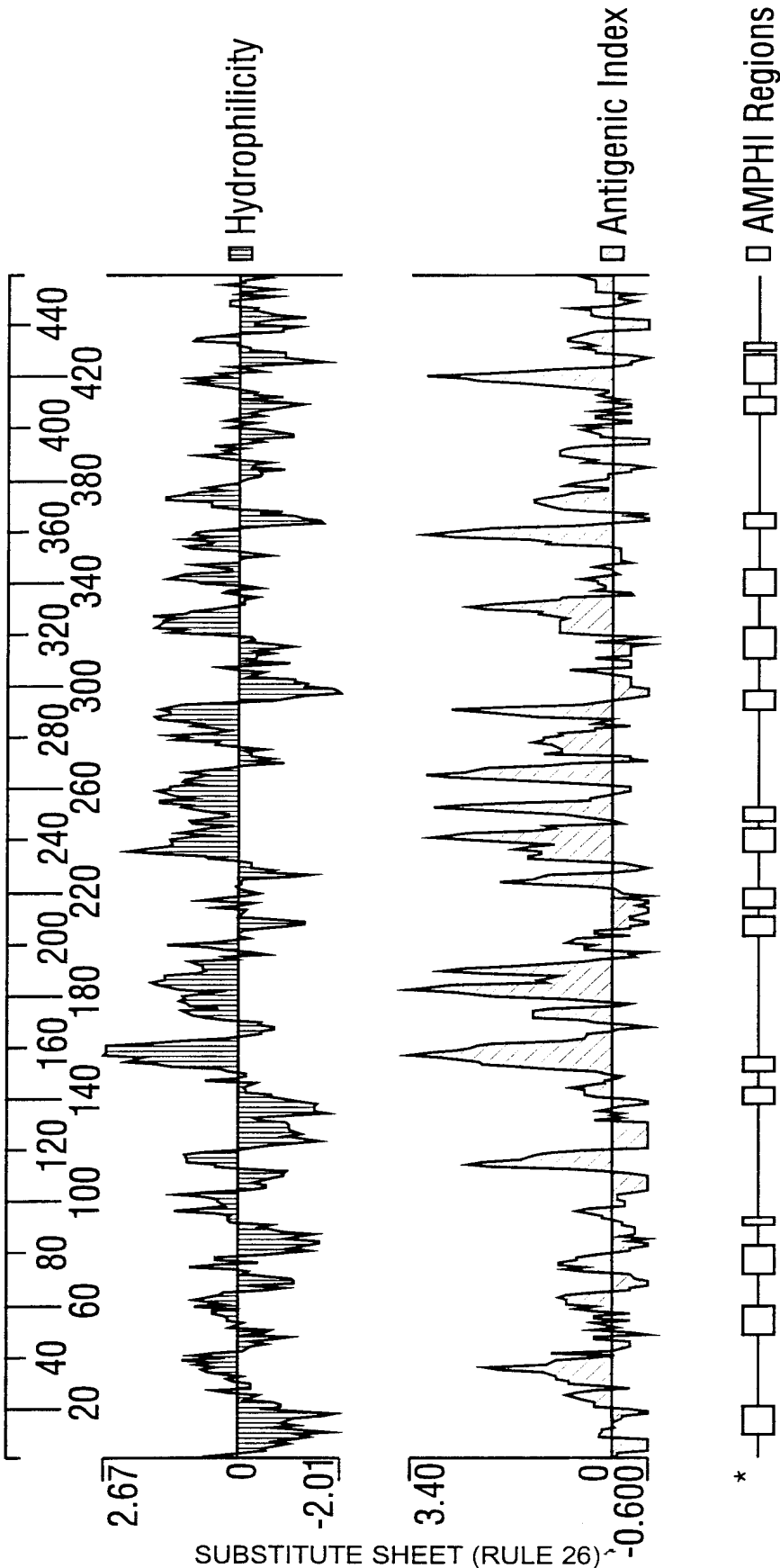
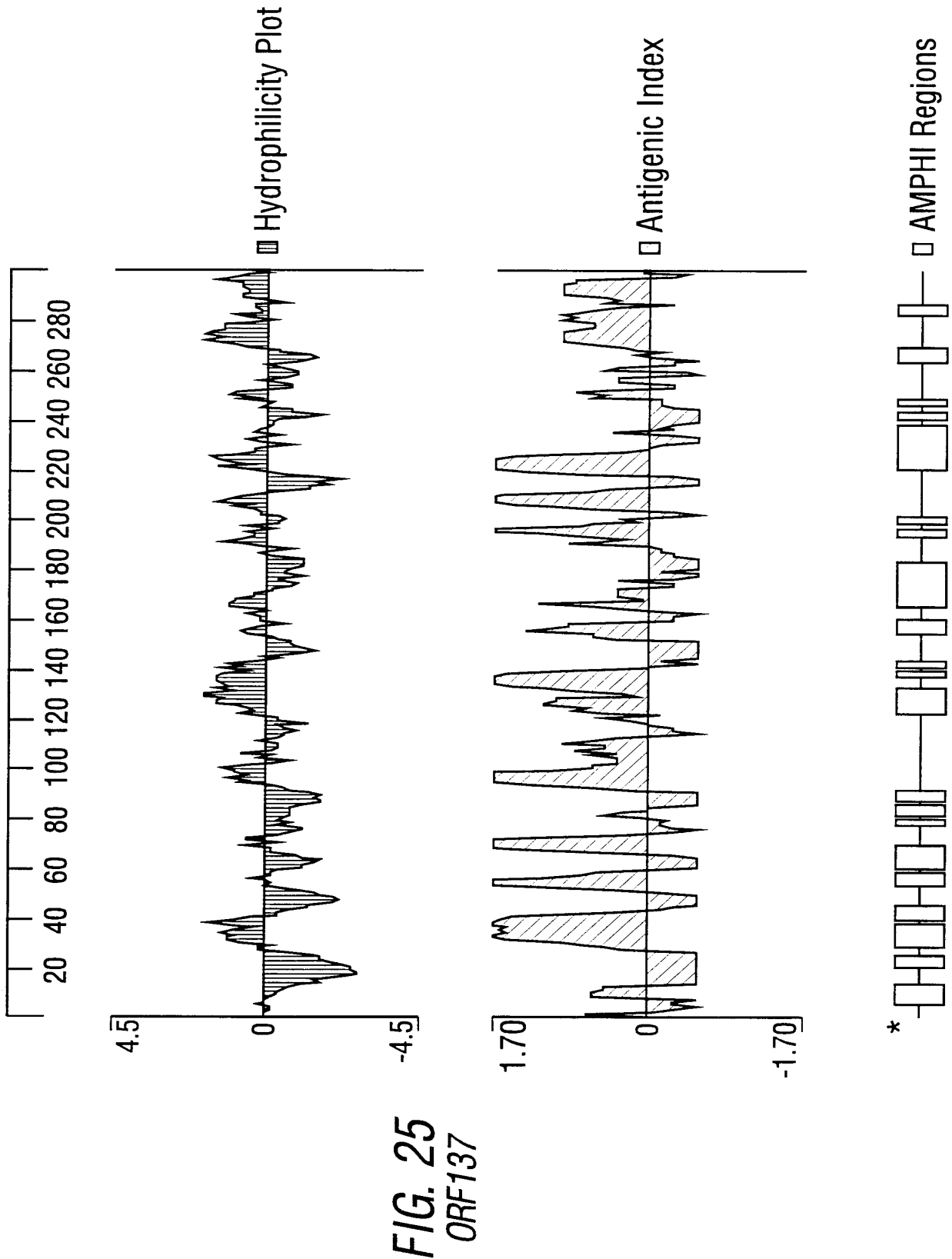


FIG. 24  
ORF132

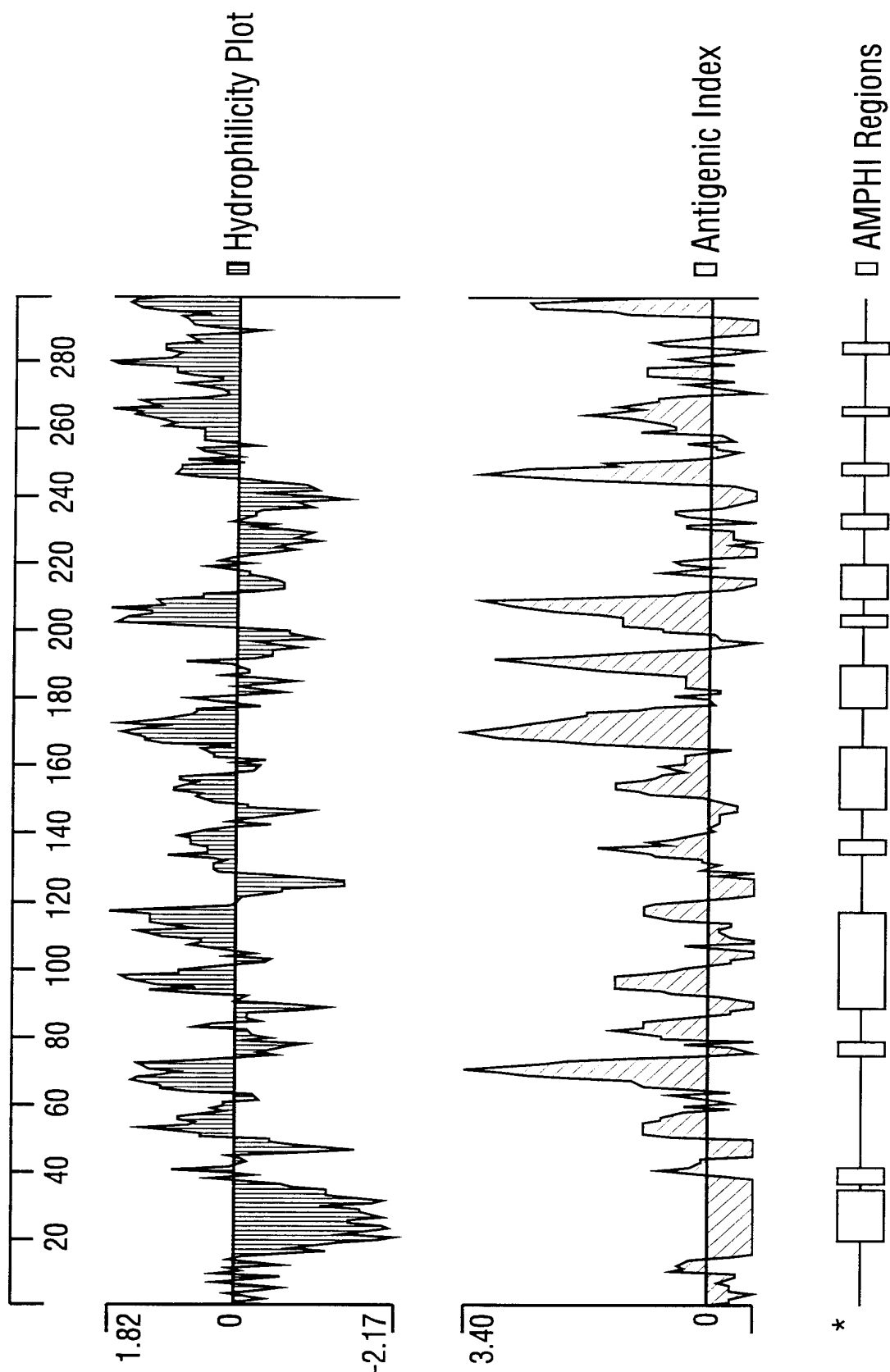






27/51

FIG. 26  
ORF138



28/51

FIG. 27  
ORF143

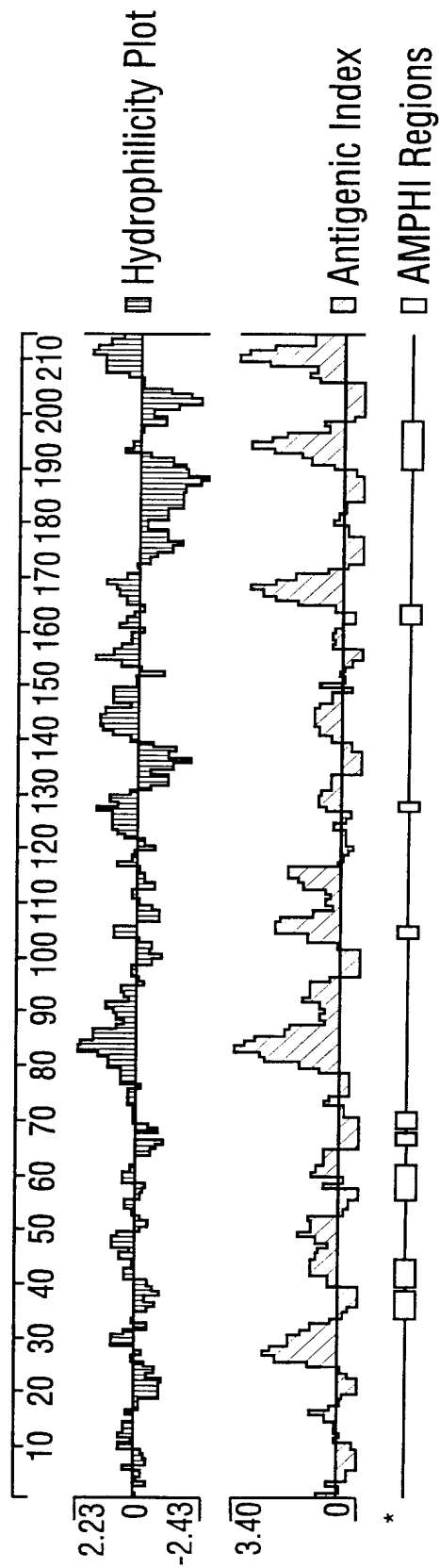
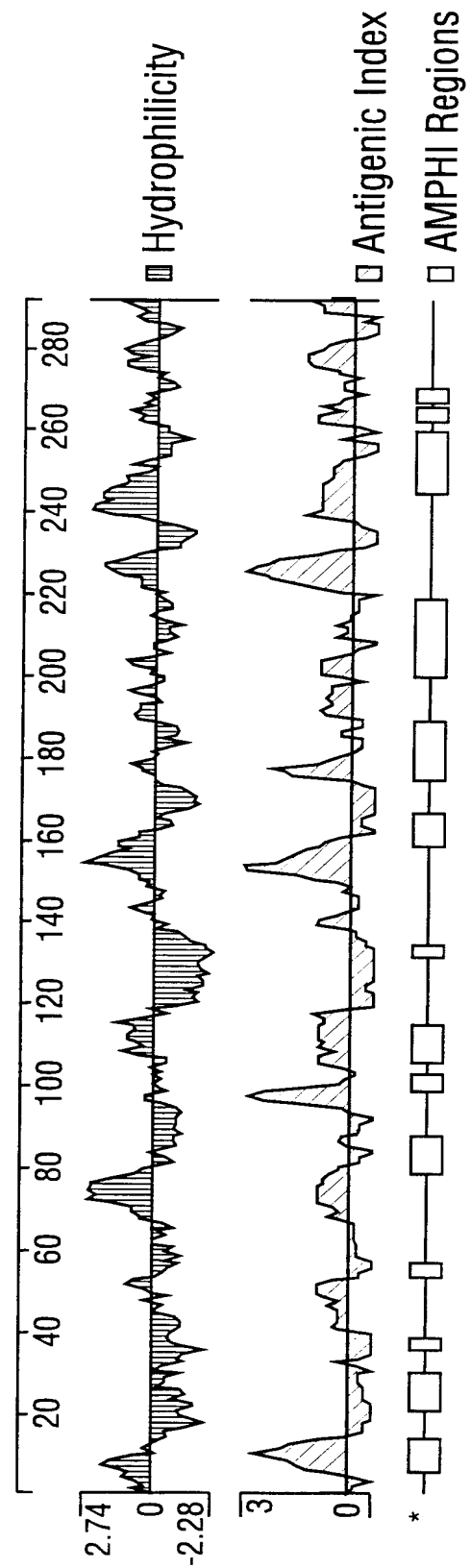


FIG. 28  
ORF147



29/51

**FIG. 29**  
NspA sequences

	Signal Peptide					Loop 1				
	-10	1	10	20	30	40				
NM 608 B	MKKALATLIA	LALPAAALAE	GASGFYVQAD	AAHAKASSSL	<u>GSAKGFSPRI</u>	SAGYRINDL				
NM NG6/88+	-----	-----	-----	-----	-----	-----				
NM CU385+	-----	-----	-----	-----	-----	-----				
NM 8047+	---A---	-----	-----	-----	-----	-----				
NM NGP165-	-----	-----	-----	-----	-----	-----				
NM MC58-	-----	-----	-----	-----	-----	-----				
NM M986-	-----	-----	-----	-----	-----	-----				
NM BZ232-	-----	-I-----	-----	-----	-----	-----				
NM M136-	-----	-I-----	-----	-----	-----	-----				
NM NG3/88-	-----	-I-----	-----	-----	-----	-----				
NG FA1090	---A---	-----	-----	-----*	-----	-----				
NG B2	---A---	-----	-----	-----	-----	-----				

30/51

*FIG. 29 (contd.)*  
*NspA sequences*

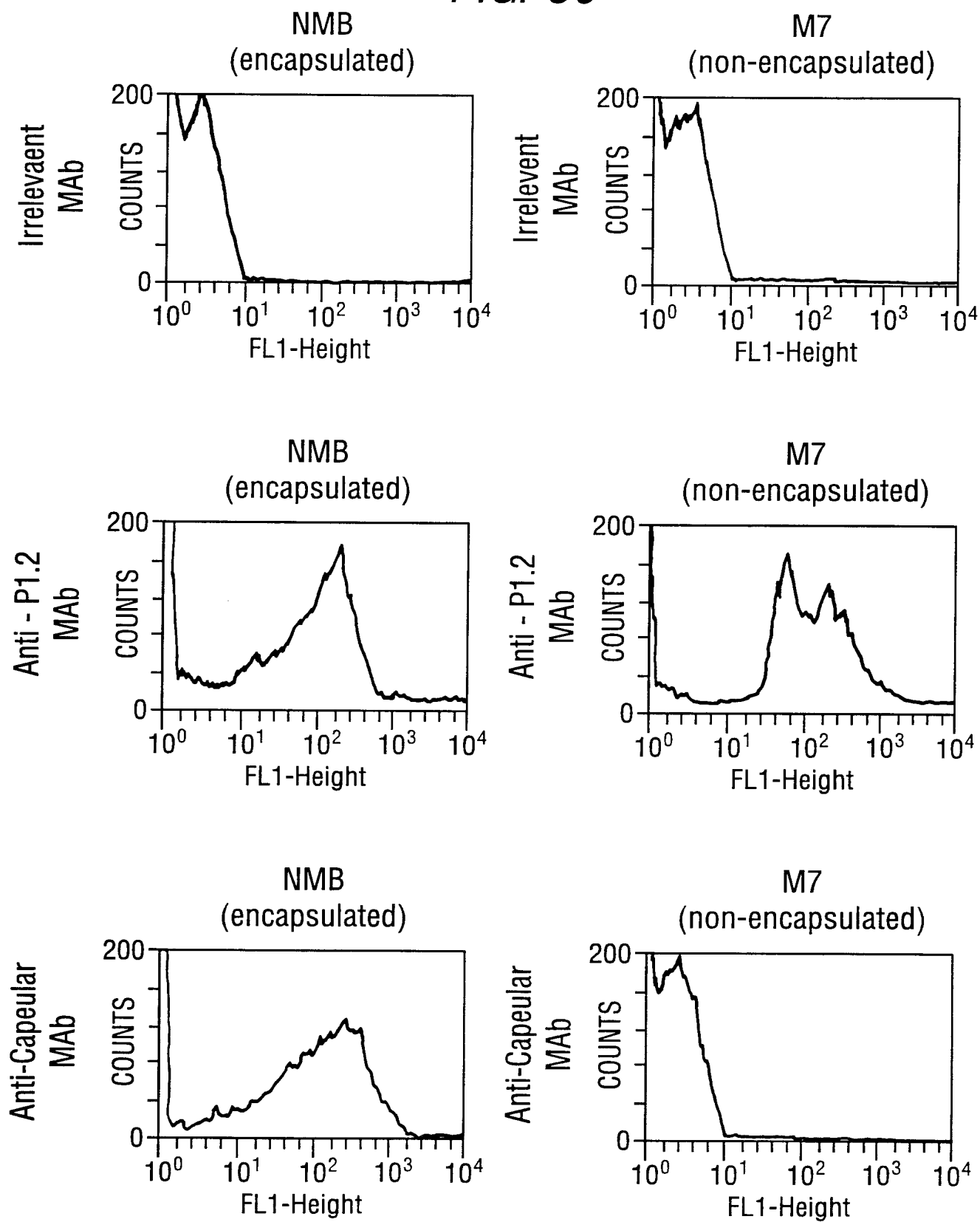
	Loop 2					Loop 3				
	50	60	70	80	90	100				
NM 608B	RFAVDYTRYK	<u>NYKAPSTDFK</u>	LYSIGASAIY	DFDTQSPVKP	YLGARLSLNR	<u>ASVDLGGSDS</u>				
NM NG6/88+	-----	-----	-----	-----	-----	-----				
NM CU385+	-----	-----	-----	-----	-----	-----				
NM 8047+	-----	-----	-----	-----	-----	-----				
NM NGP165-	-----	-----	-----	-----	-----	-----				
NM MC58-	-----	-----	-----	-----	-----	-----				
NM M986-	-----	-----	-----	-----	-----	-----				
NM BZ232-	-----	-----	-----	-----	-----	-----				
NM M136-	-----	-----	-----	-----	-----	-----				
NM NG3/88-	-----	-----	-----	-----	-----	-----				
NG FA1090	-----	---Q---	-----V-	-----F-	-----F-	---AH---				
NG B2	-----	-----	-----V-	-----F-	-----F-	---AH---				

31/51

	110	120	130	140	150
	Loop 4				
NM 608B	<u>FSQTSIGLV</u>	LTGVS	YAVTP	NVDLD	AGYR
NM NG6/88+	---	---	---	---	---
NM 8047+	---	T---	---	---	---
NM CU385+	---	---	---	---	A---
NM NGP165-	---	---	---	---	---
NM MC58-	---	---	---	---	A---
NM M986-	---	---	---	---	---
NM BZ232-	---	T---	---	---	A---
NM M136-	---	T---	---	---	---
NM NG3/88-	---	T---	---	---	A---
NG FA1090	---	K-A---	---	V---	A---
NG B2	---	K-A---	---	V---	A---

FIG. 29 (contd.)  
NspA sequences

32/51

**FIG. 30**

33/51

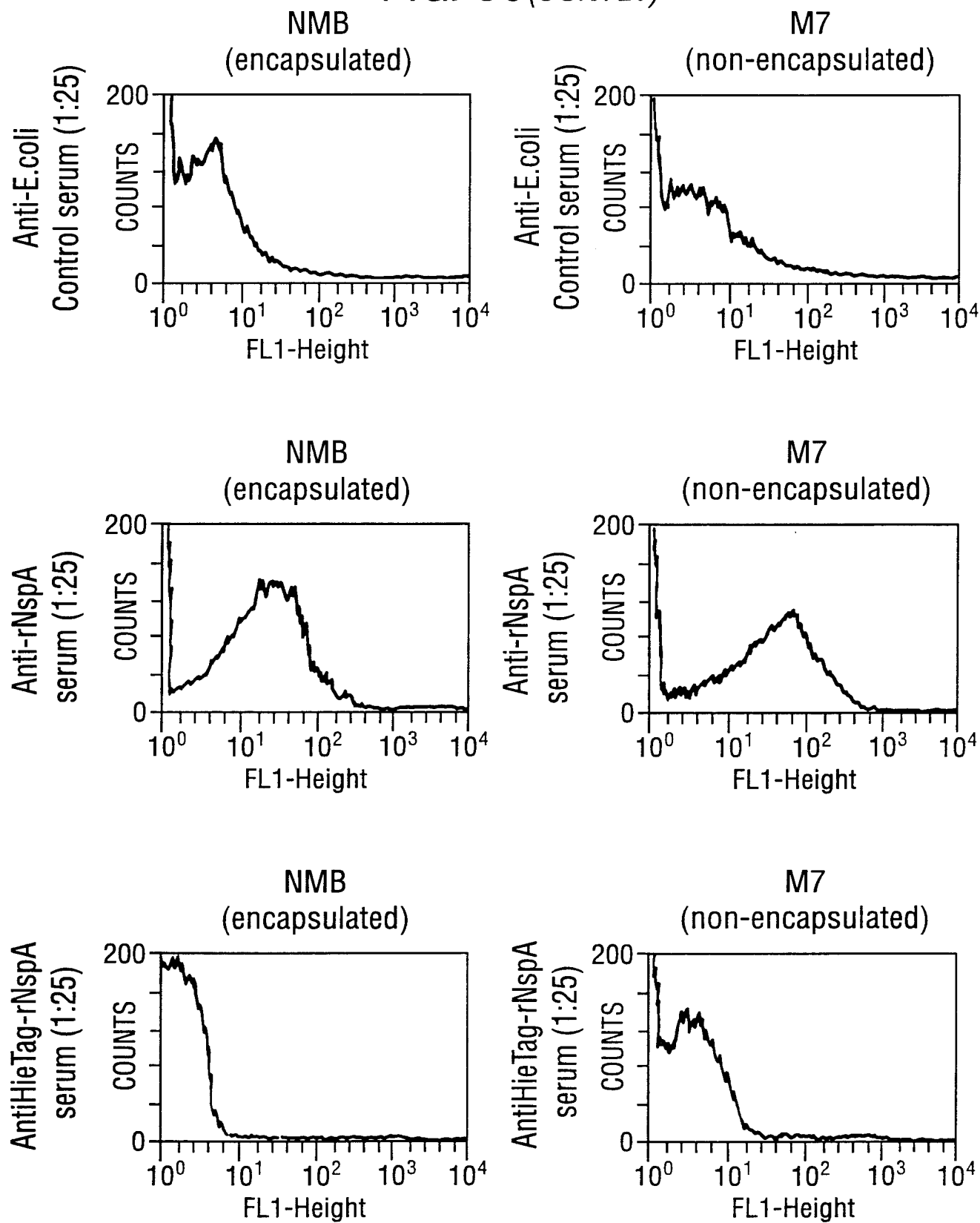
*FIG. 30(CONTD.)*



FIG. 31A

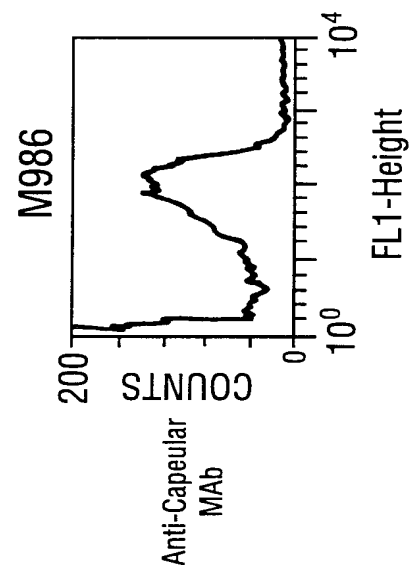
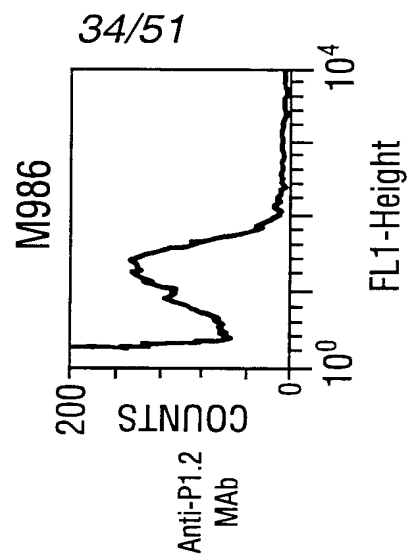
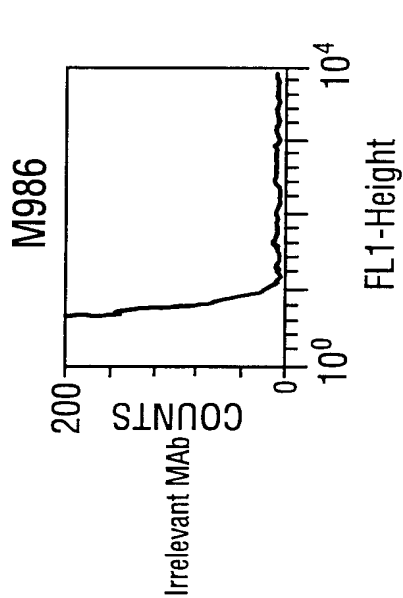
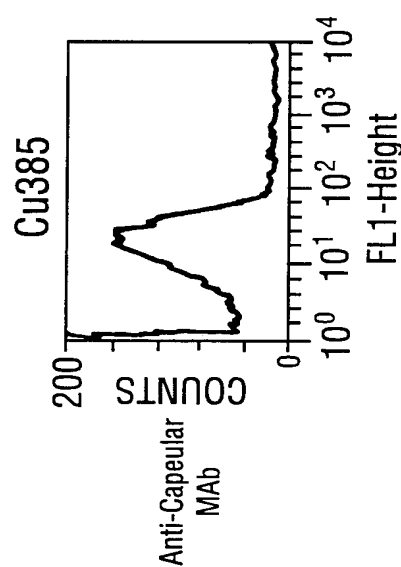
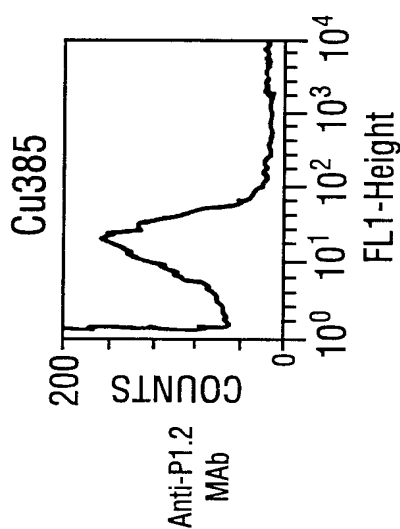
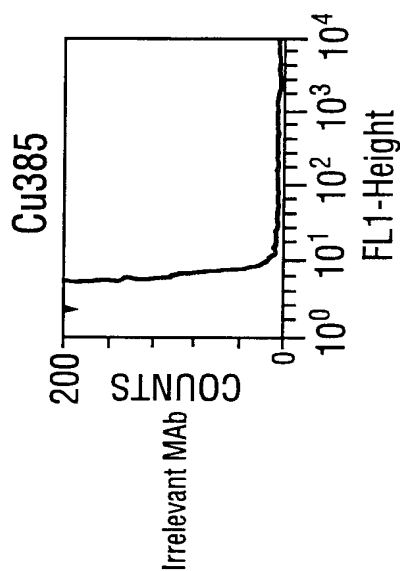
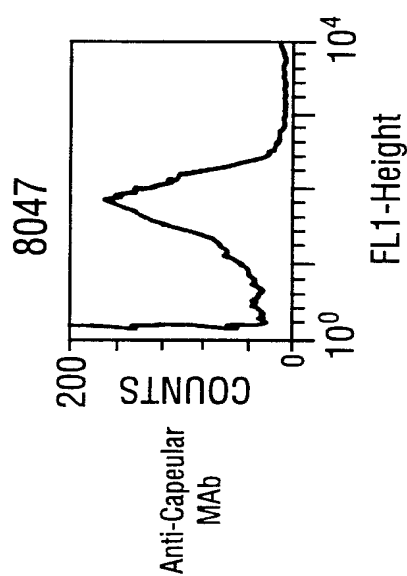
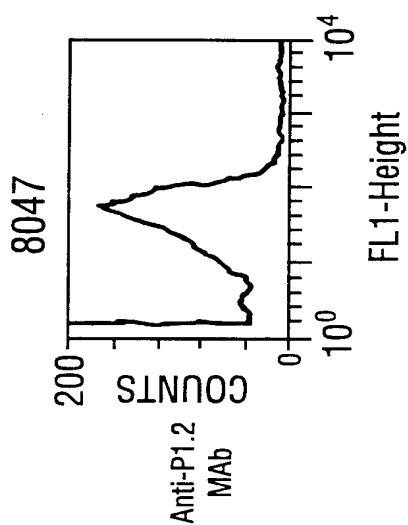
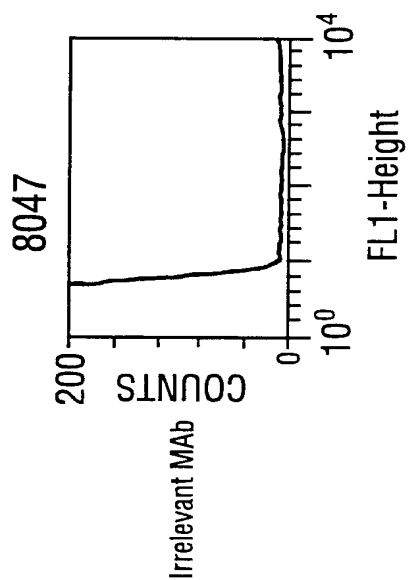
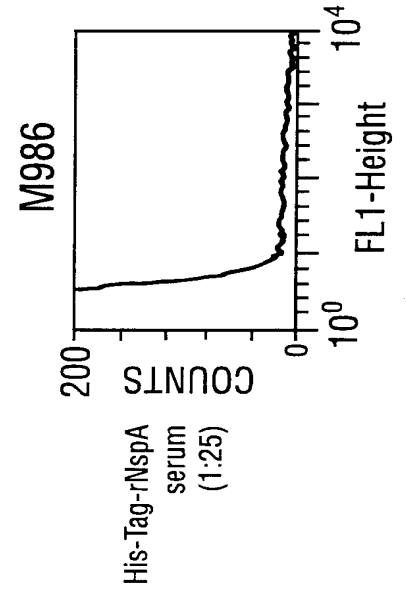
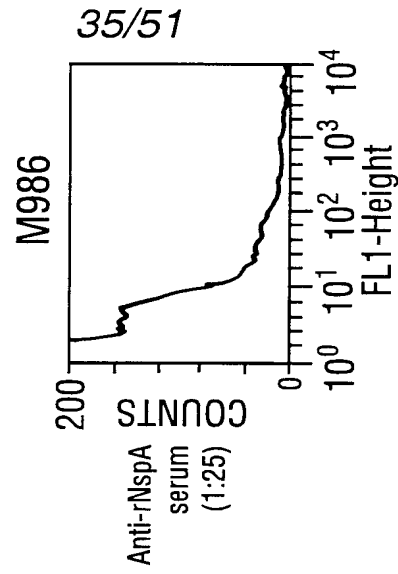
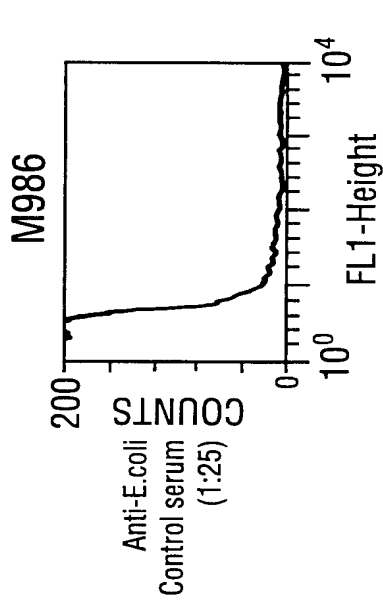
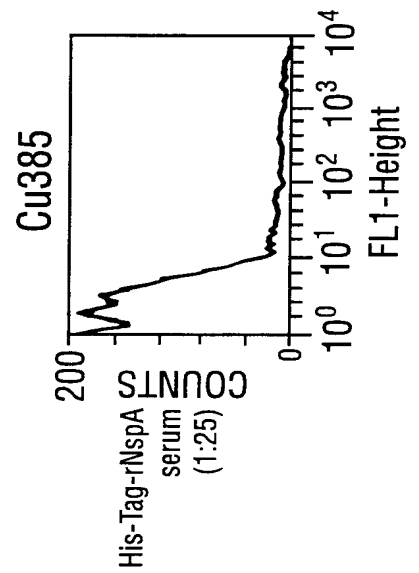
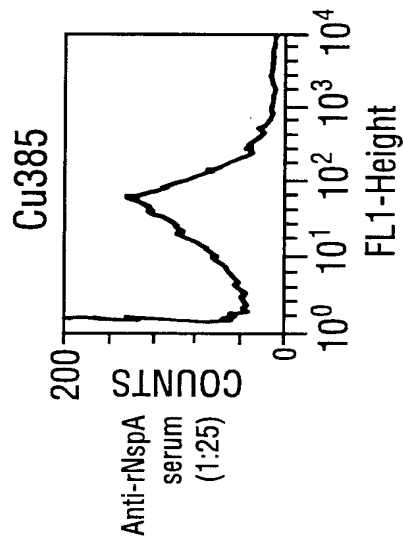
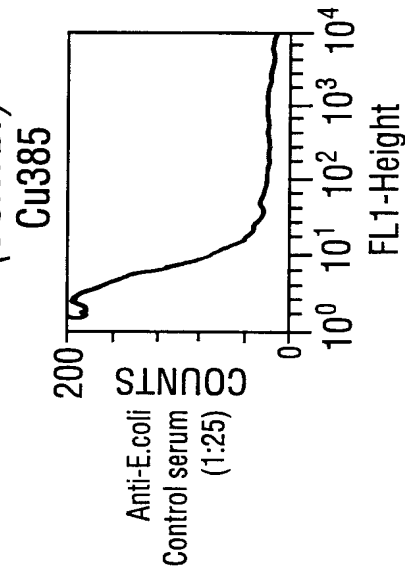
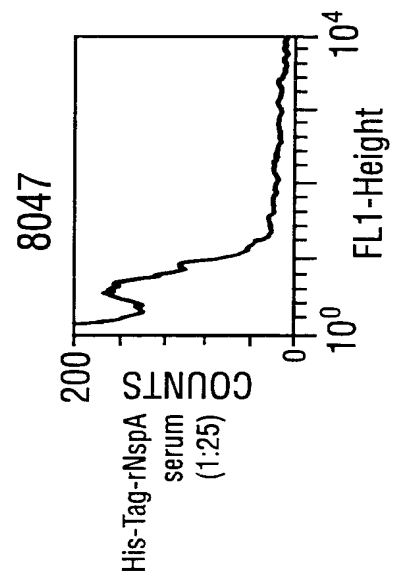
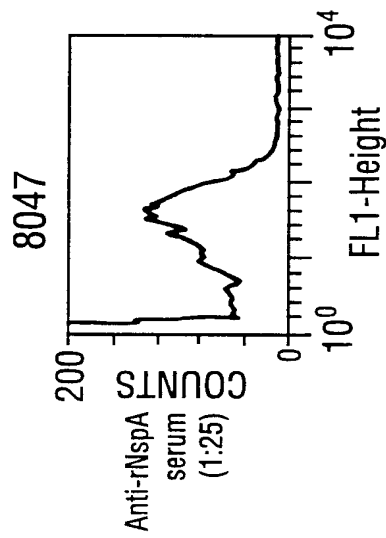
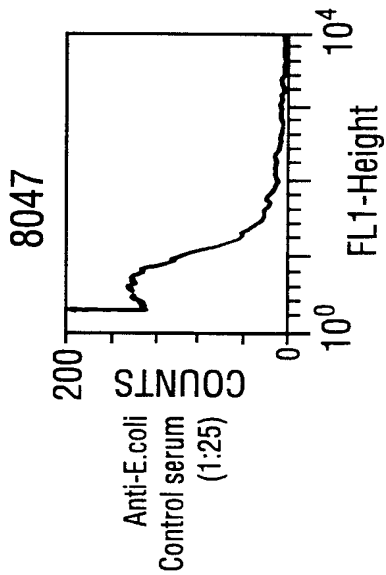


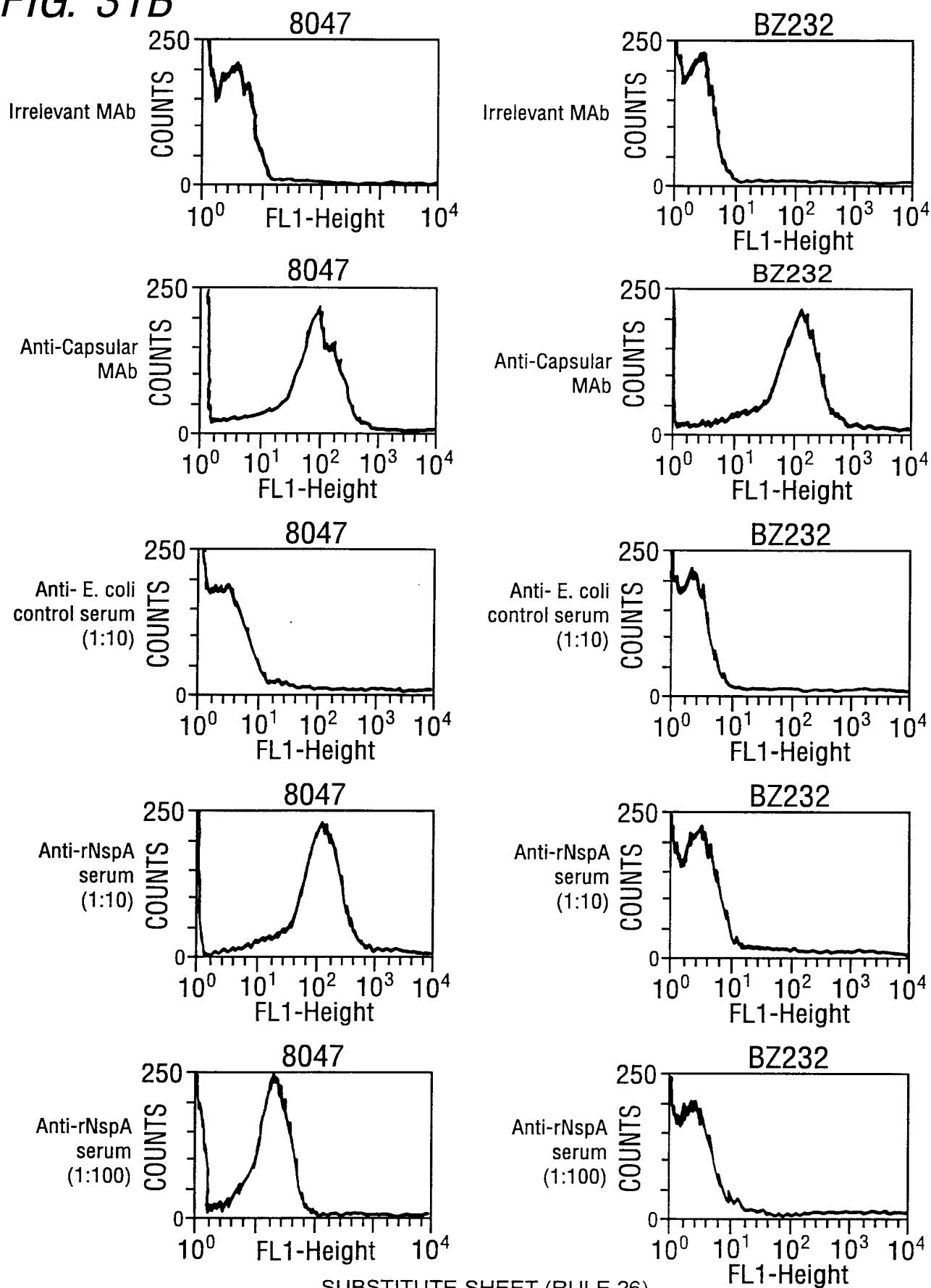
FIG. 31A(CONTD.)

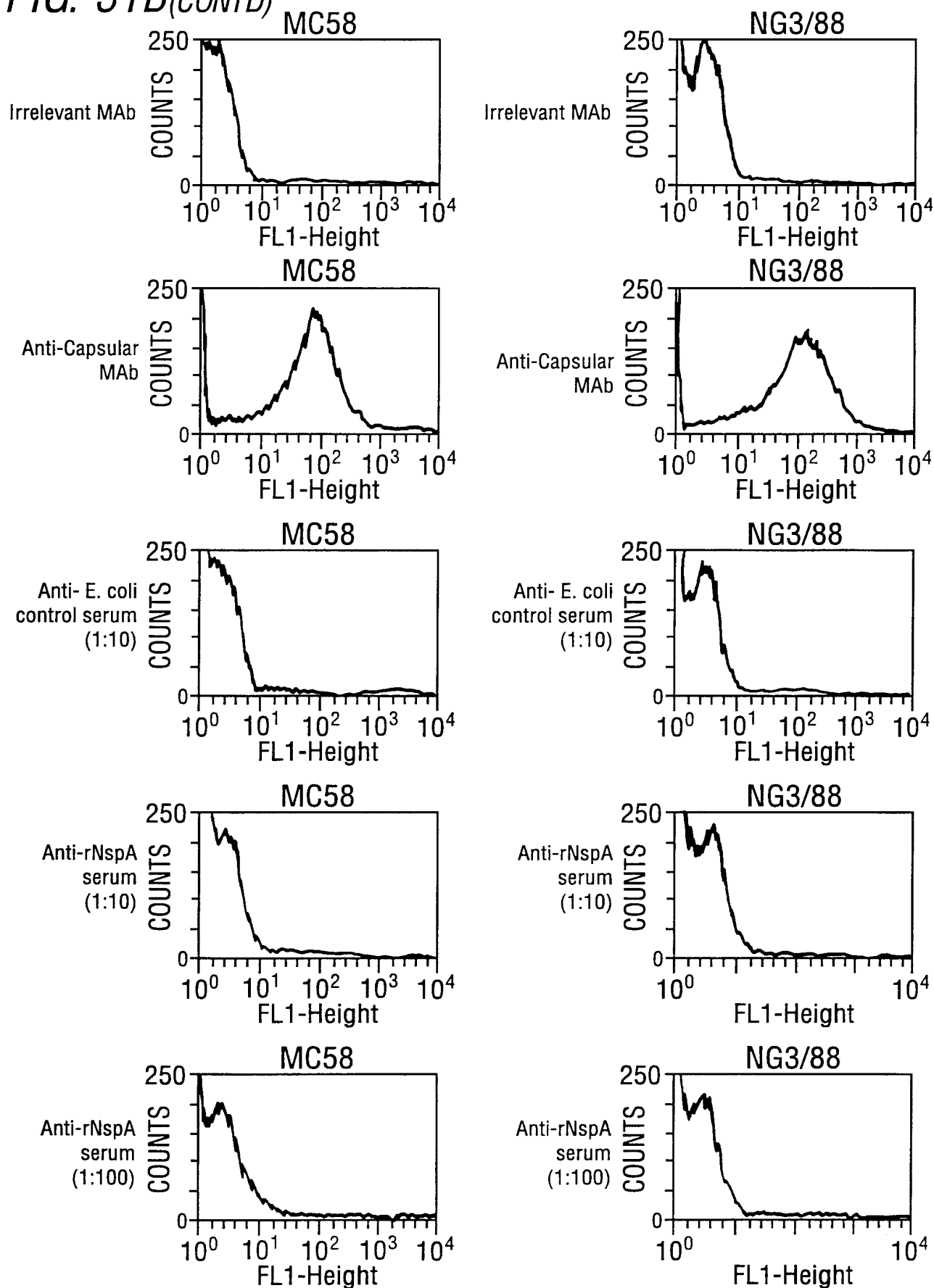


35/51

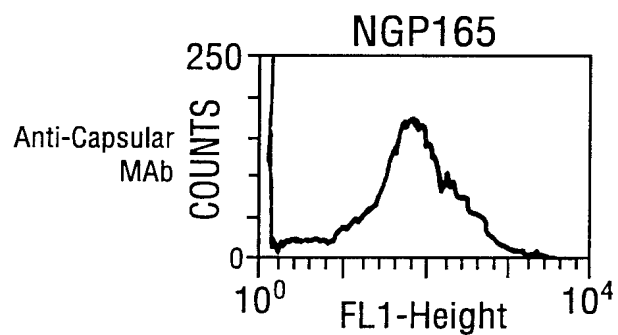
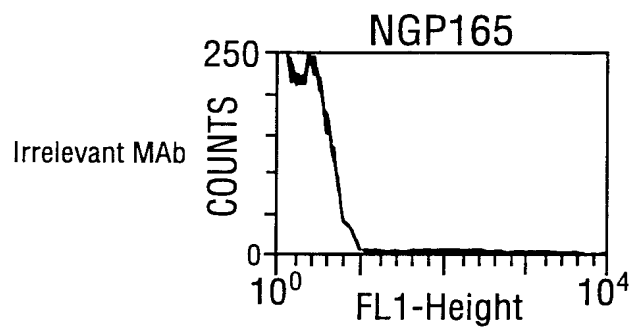
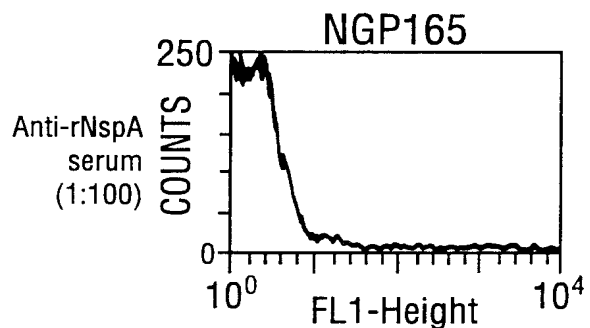
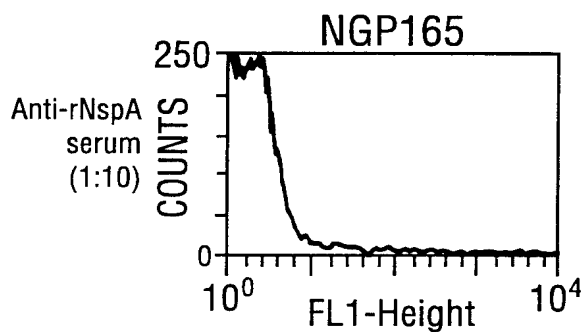
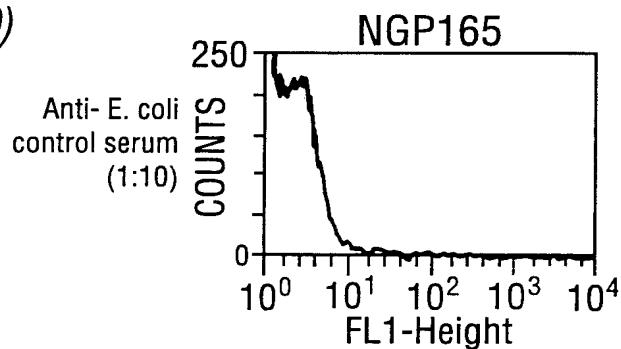
FIG. 31B

36/51



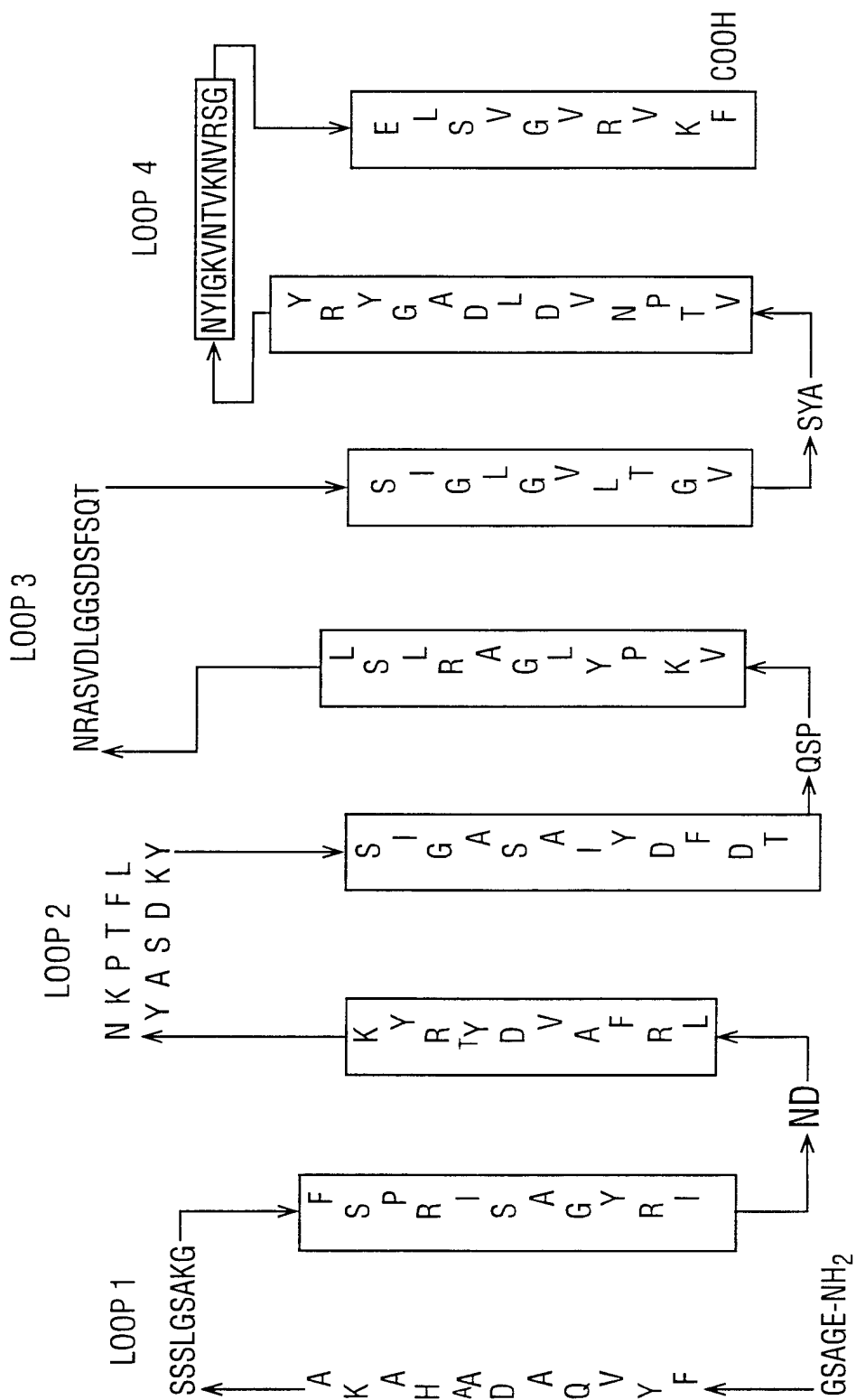
**FIG. 31B**(*CONTD*) 37/51

38/51

*FIG. 31B(CONTD)*

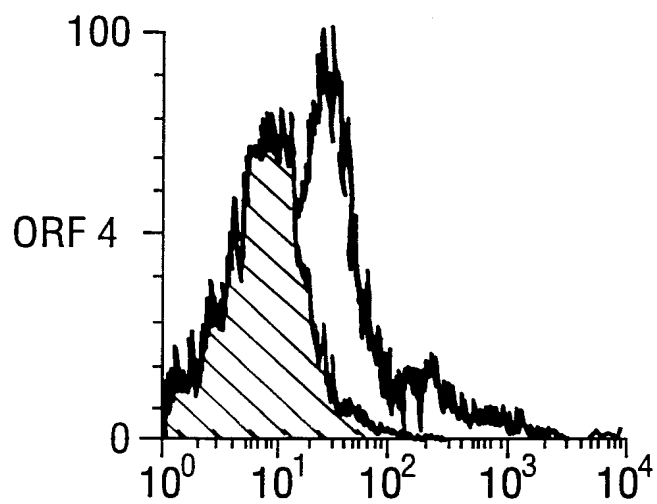
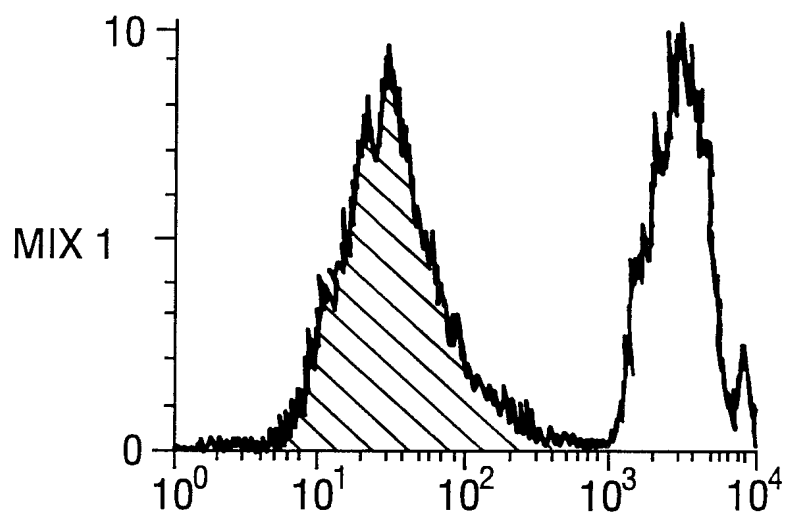
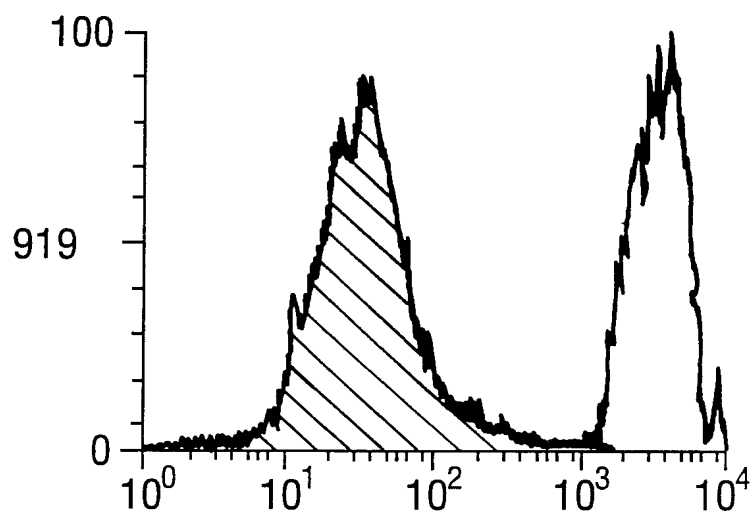
39/51

FIG. 32

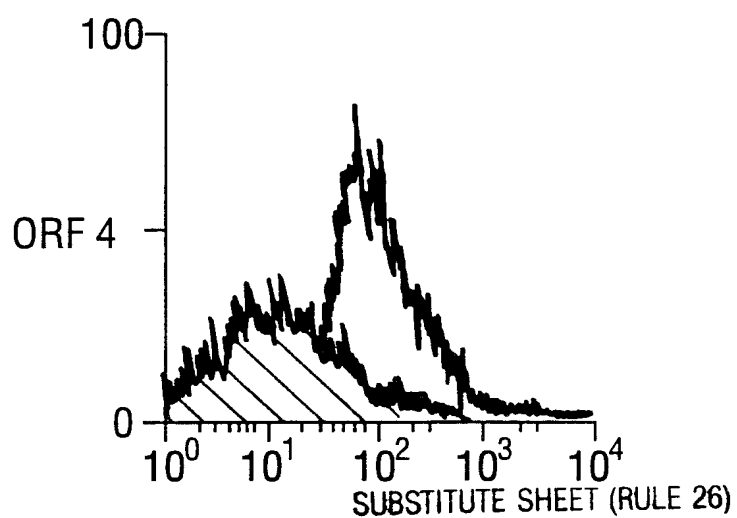
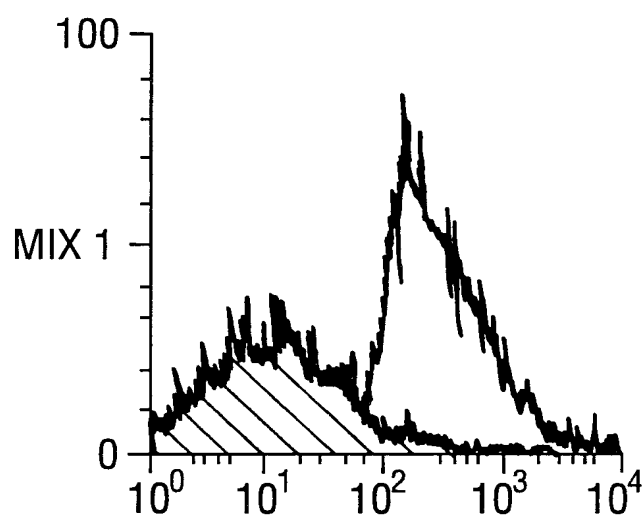
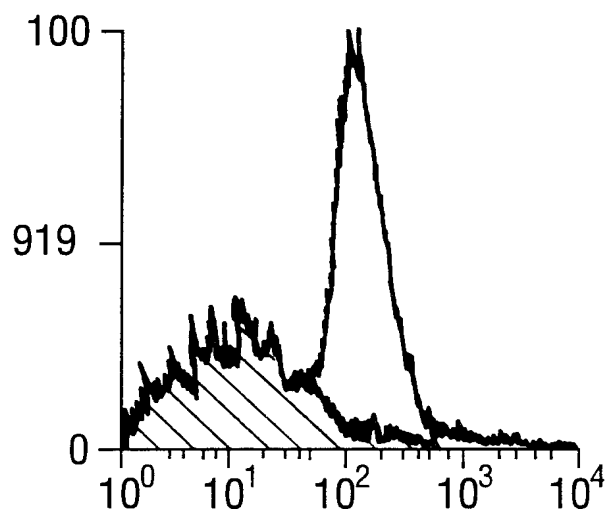


40/51

FIG. 33A



41/51  
**FIG. 33B**  
2996

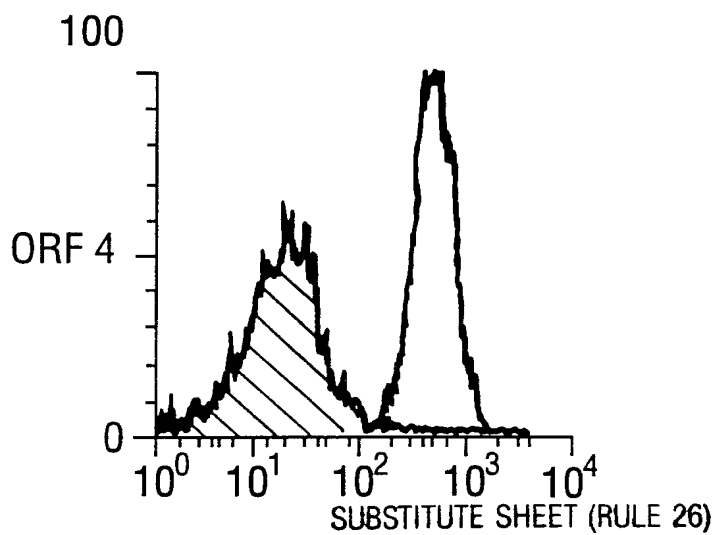
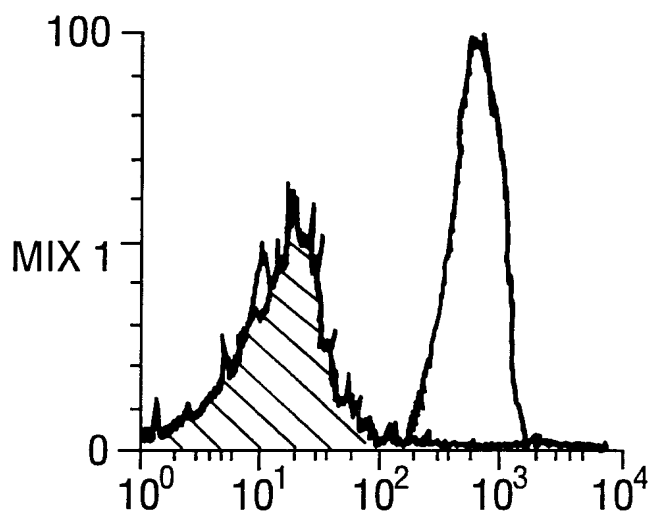
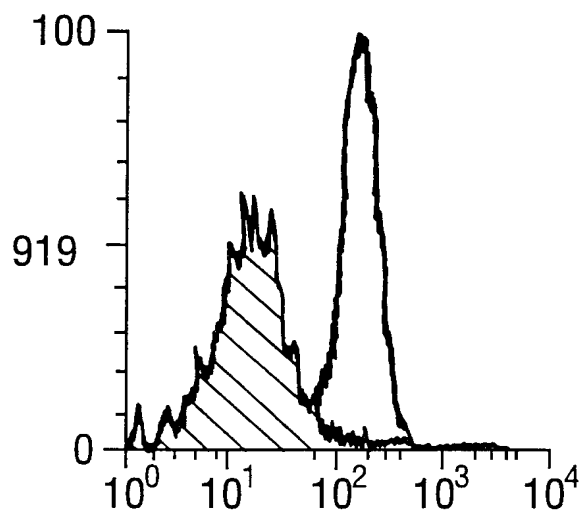




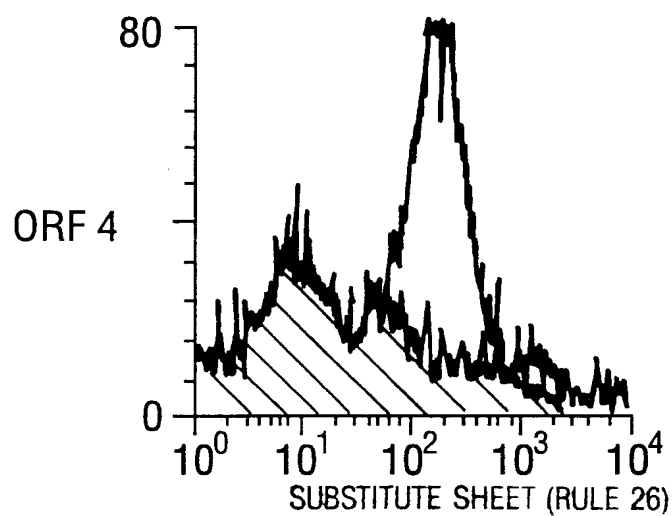
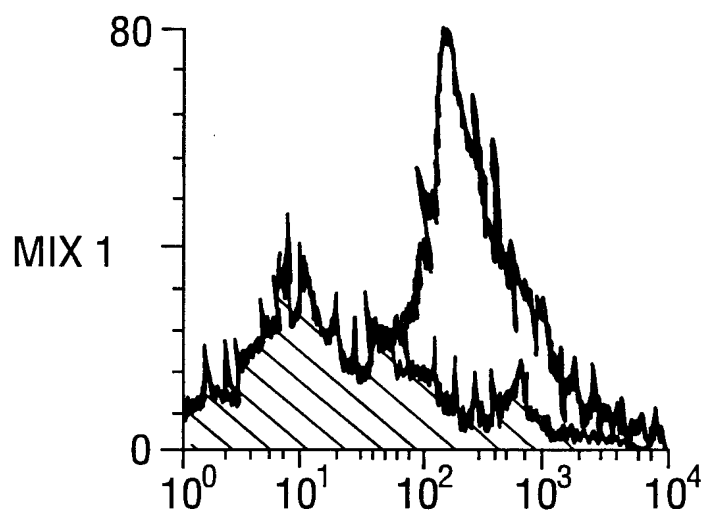
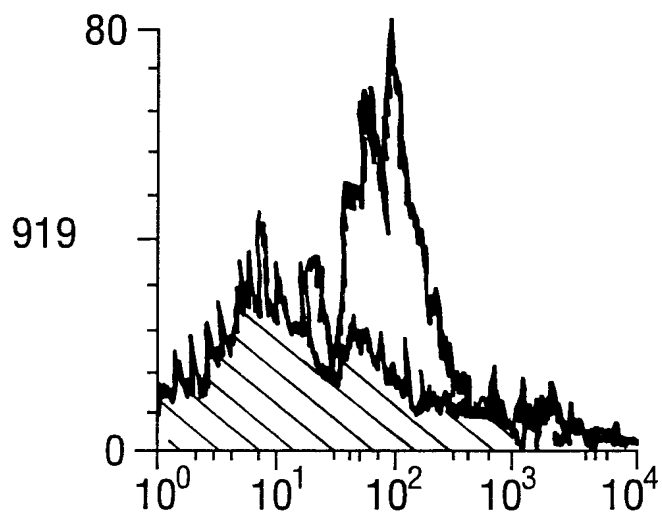
42/51

*FIG. 33B(CONTD.)*

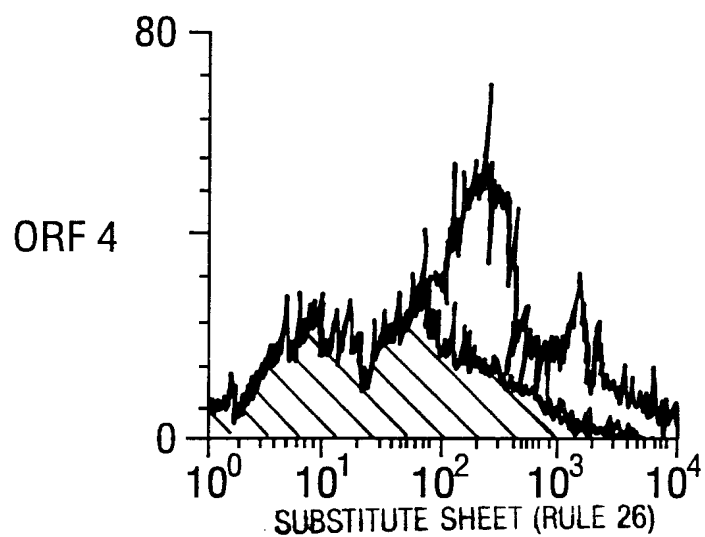
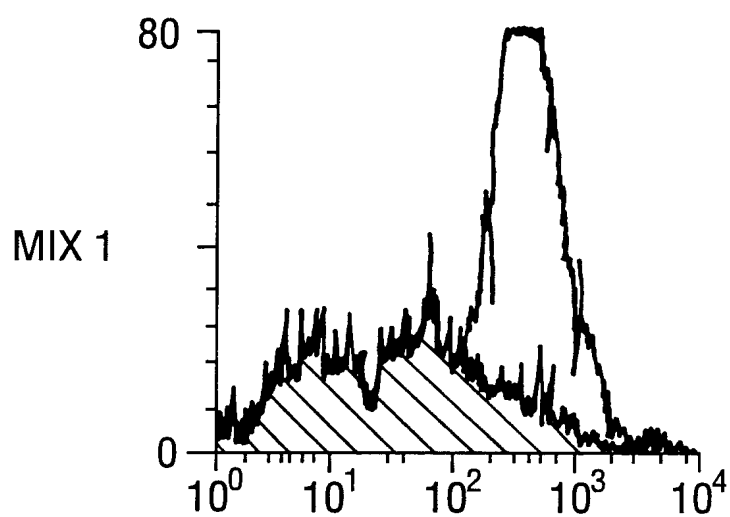
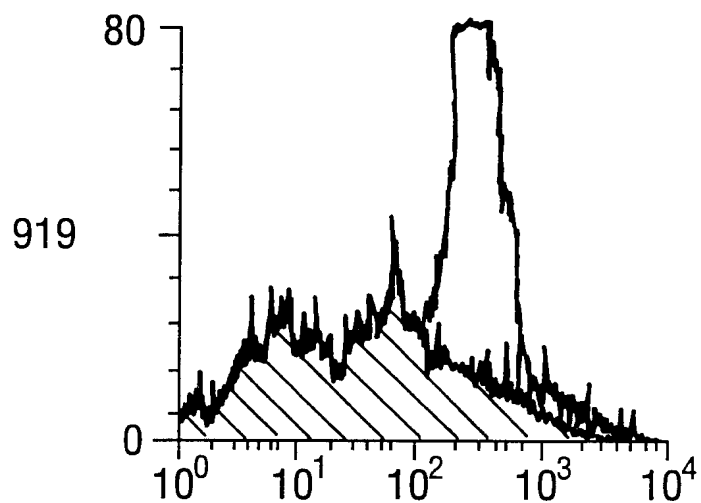
H44776



43/51  
*FIG. 33B(CONTD.)*  
MC58



44/51  
*FIG. 33B(CONTD.)*  
1000

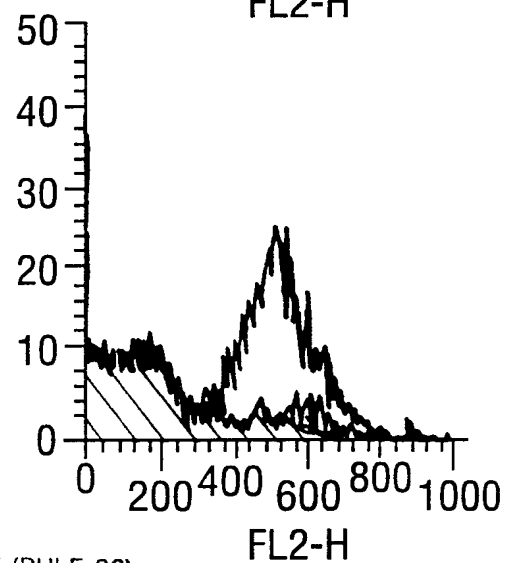
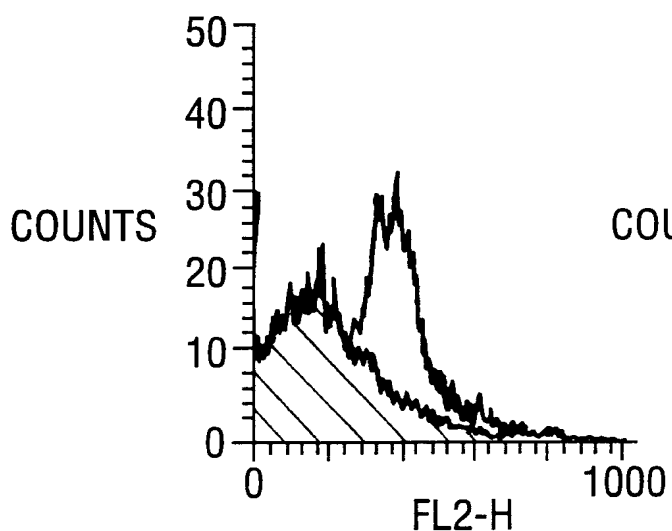
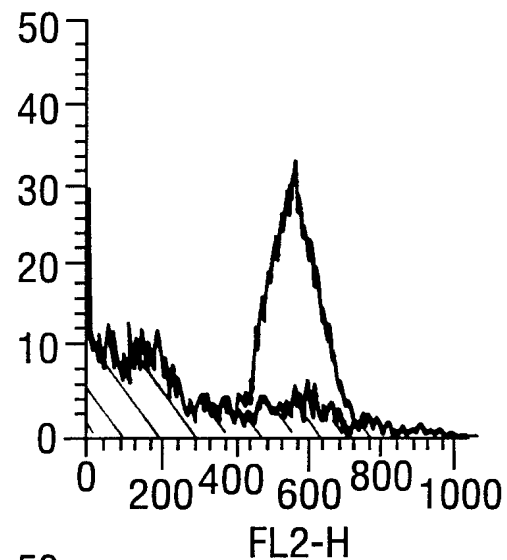
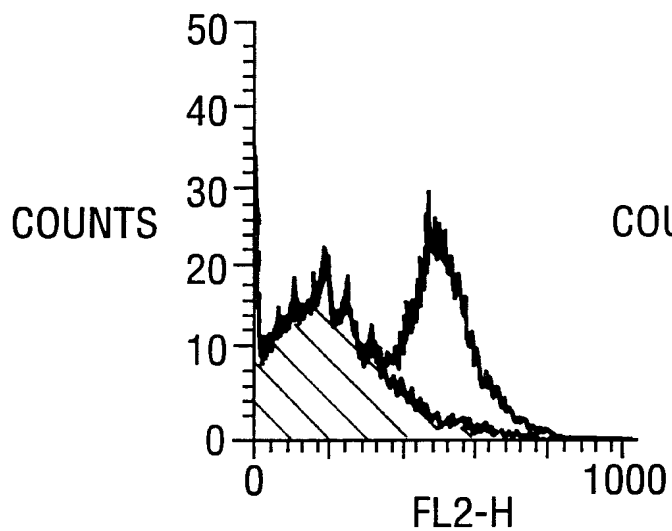
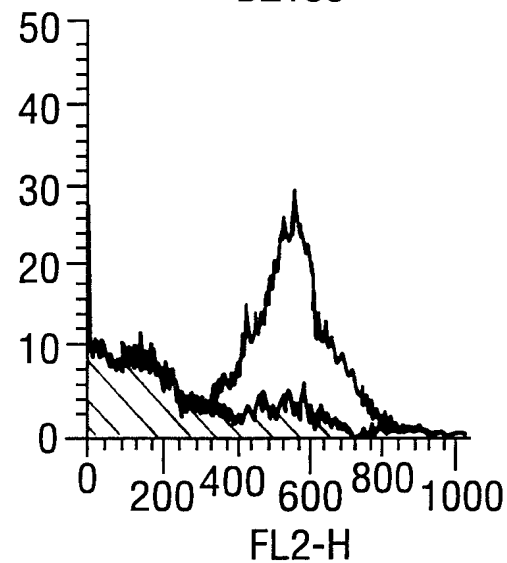
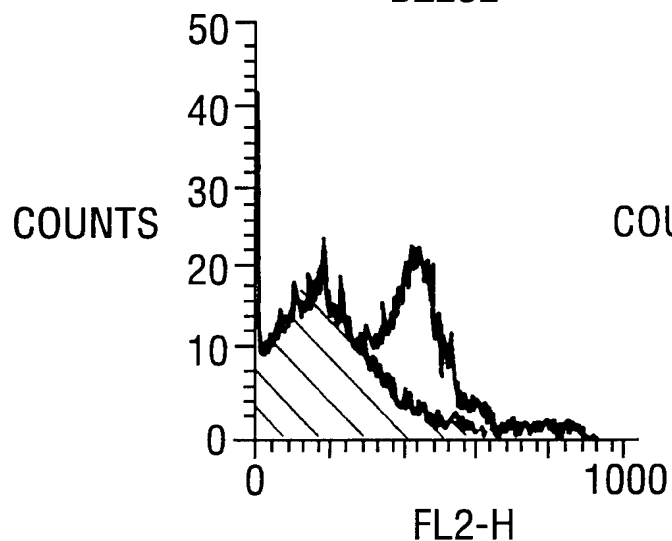


45/51

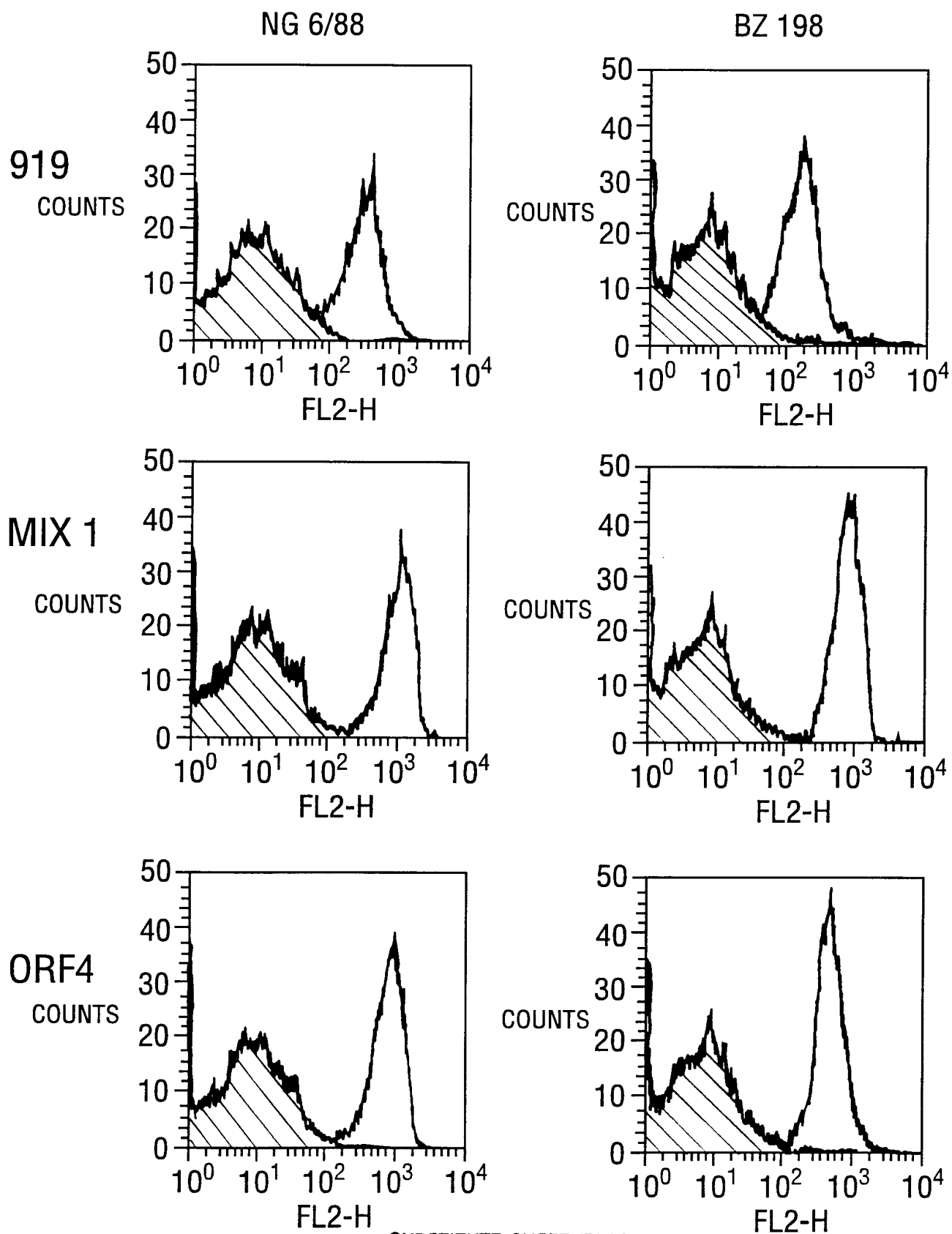
**FIG. 33B(CONTD.)**

BZ232

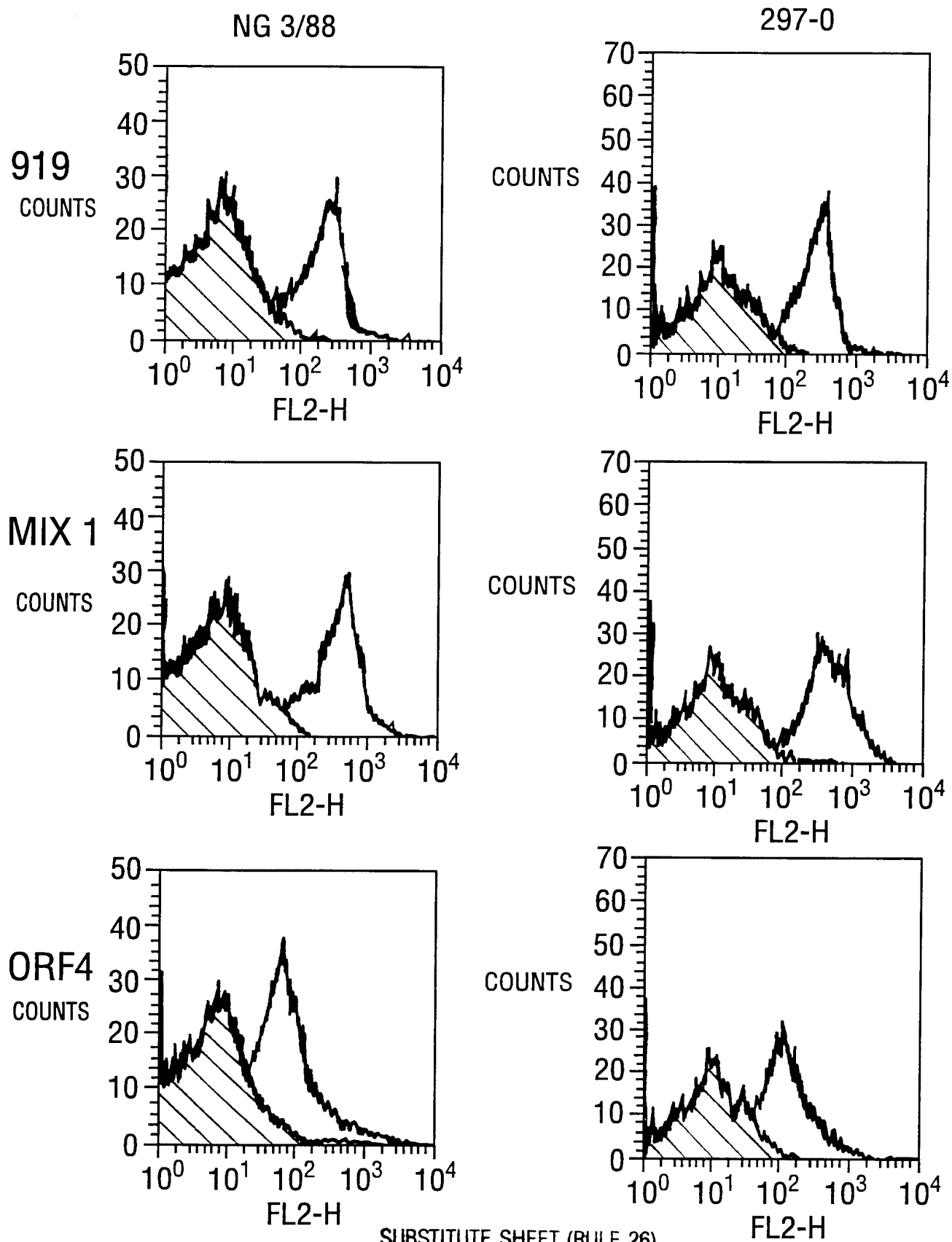
BZ133



46/51

*FIG. 33B(CONTD.)*

47/51

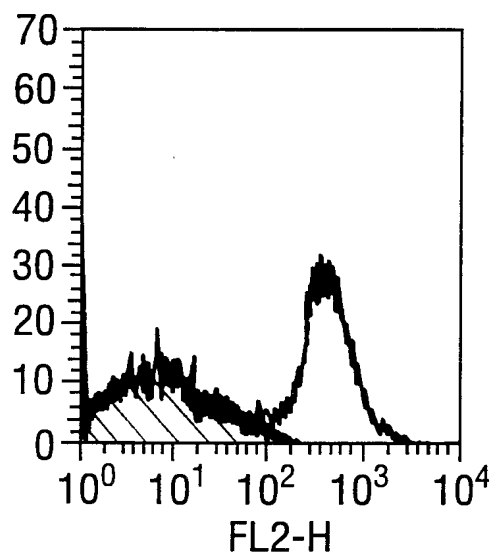
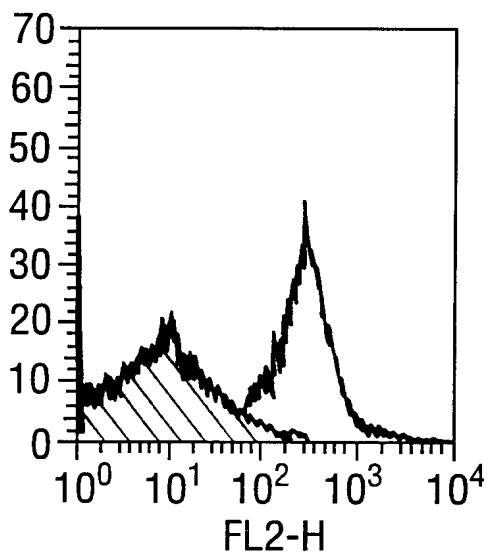
*FIG. 33B(CONTD.)*

48/51

**FIG. 33B(CONTD.)**

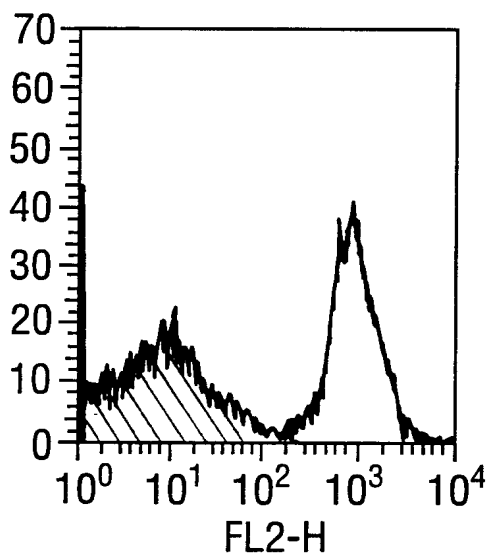
BZ147

BZ 169

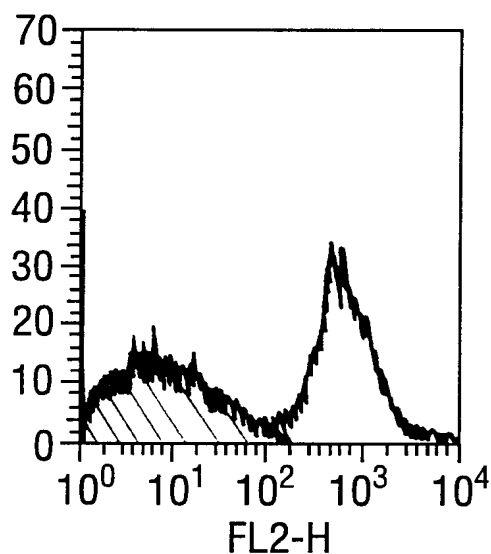


MIX 1

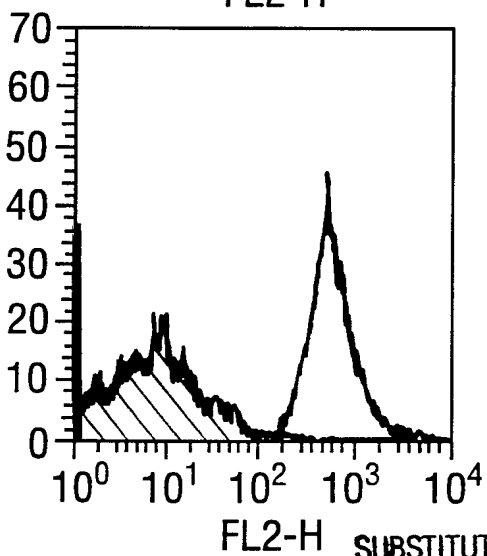
COUNTS



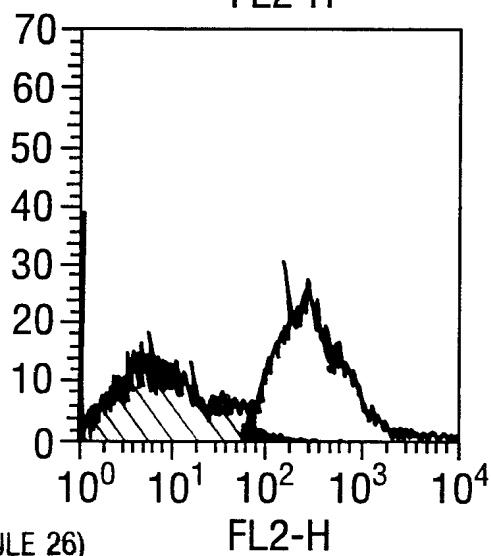
COUNTS

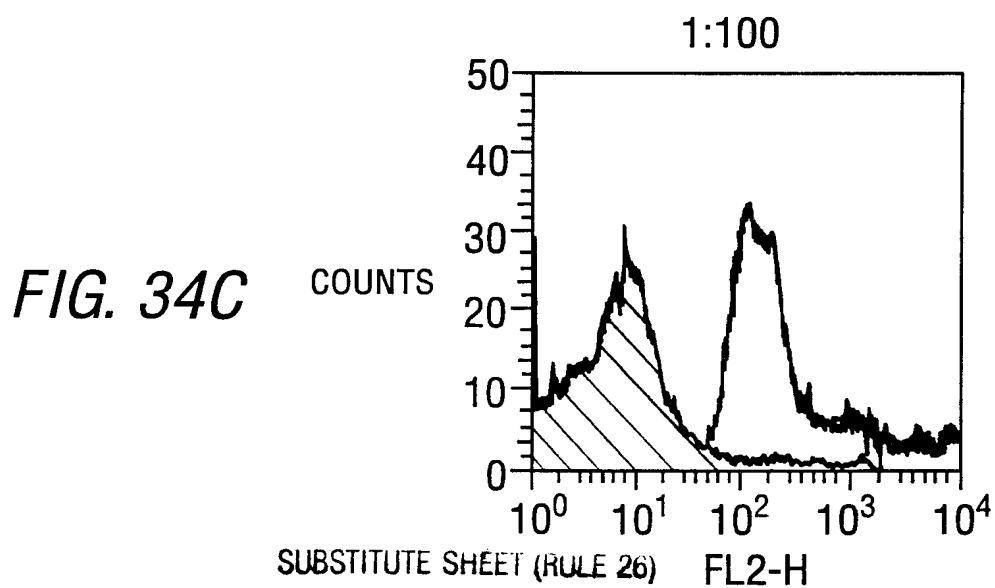
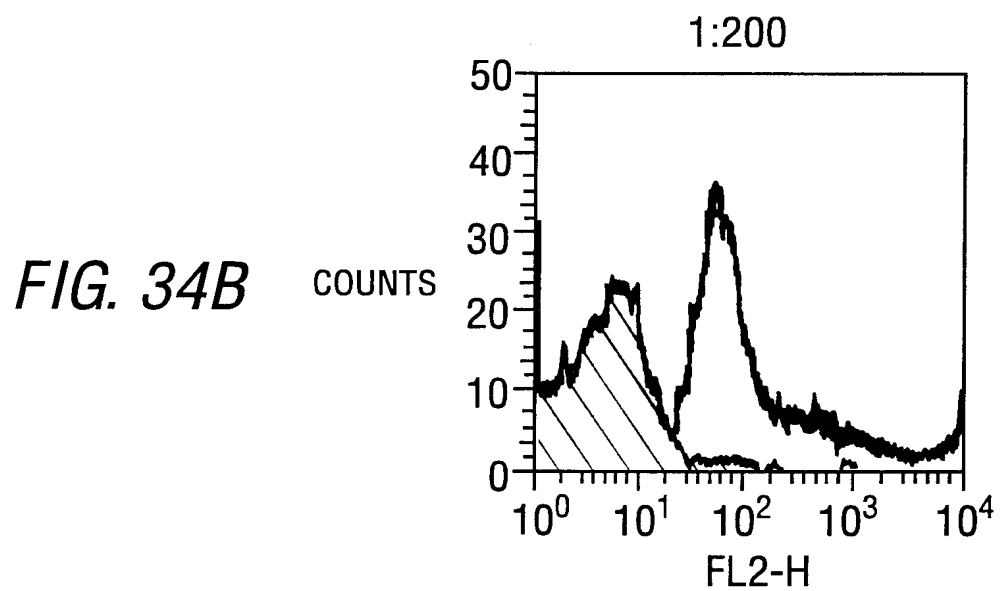
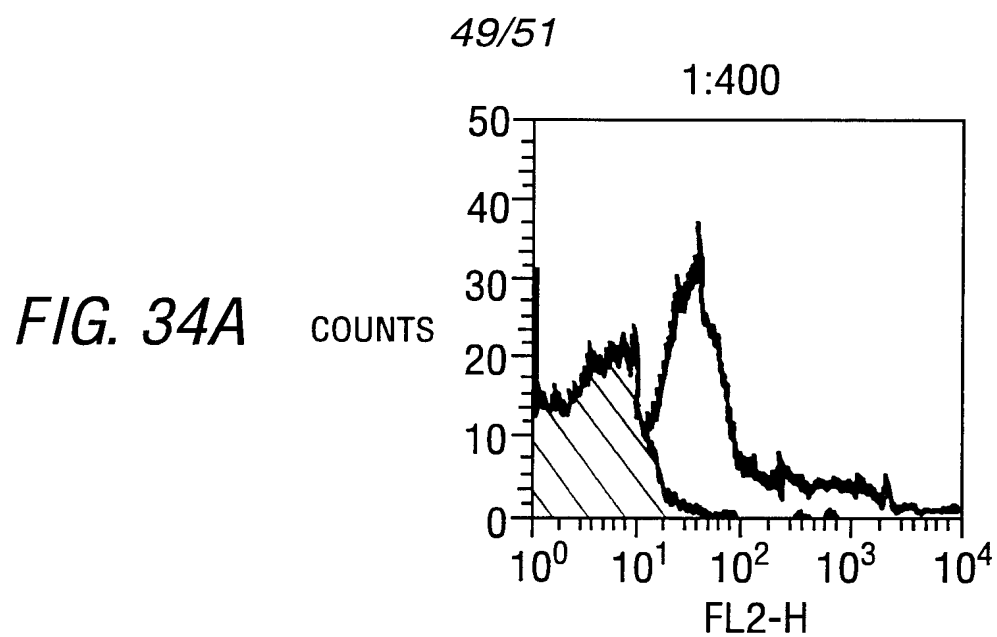


ORF4  
COUNTS



COUNTS







50/51

FIG. 35

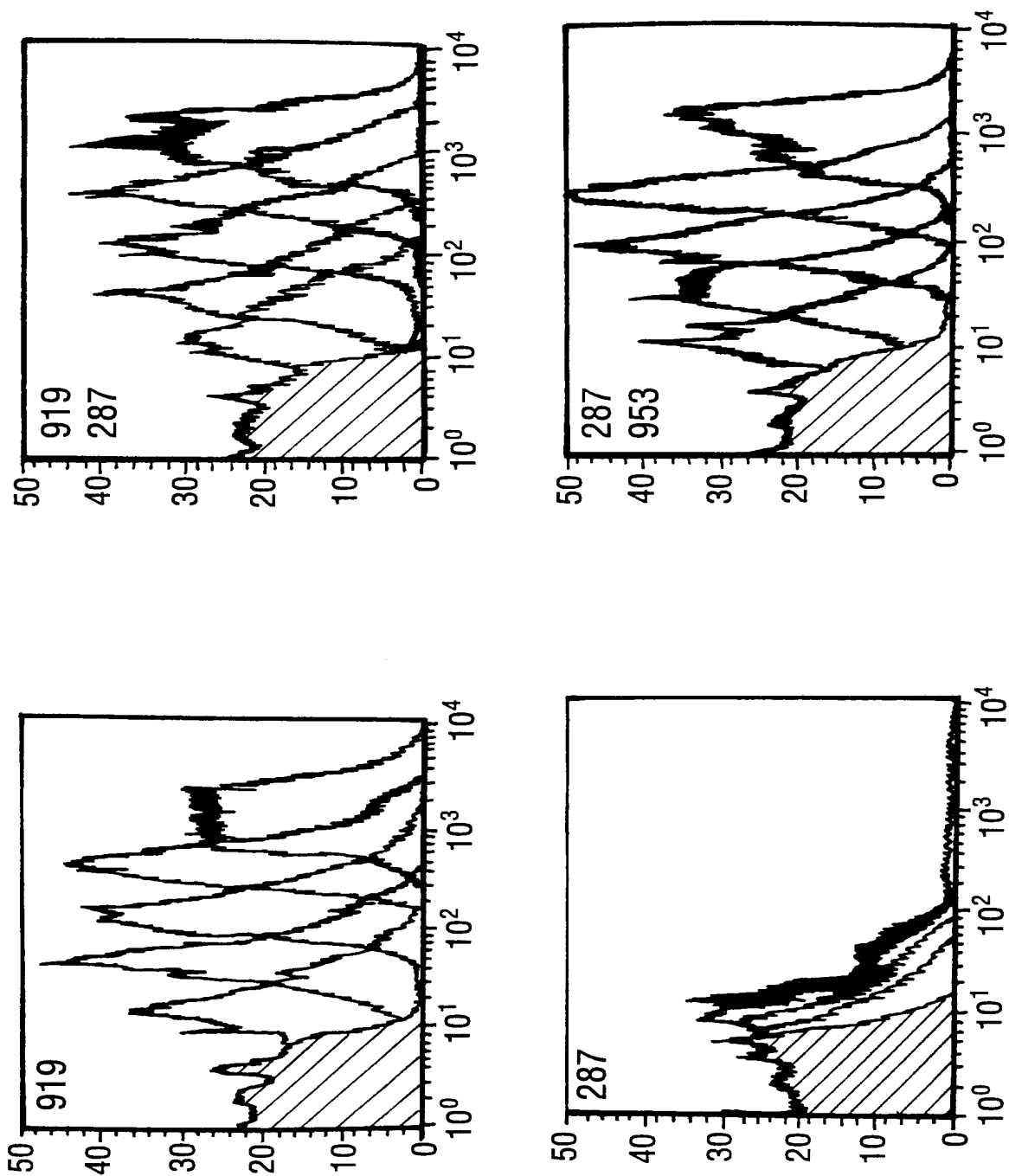


FIG. 35(CONTD.)

